THE EPIDEMIOLOGY & MOLECULAR BASIS OF FLUOROQUINOLONE RESISTANT & SUSCEPTIBLE ISOLATES OF *CAMPYLOBACTER COLI*

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ABBREVIATIONS

Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
bp	base pair(s)
C.	*Campylobacter*
°C	degrees Celsius
$CHCl_3$	chloroform
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram(s)
GBS	Guillain-Barré Syndrome
Gln	glutamine
H_20	water
hr	hour(s)
Ile	Isoleucine
kb	kilobase(s)
M	molar
MCS	multiple cloning site

mg	milligram(s)
$MgCl_2$	magnesium chloride
MIC(s)	minimum inhibitory concentration(s)
min	minute(s)
µl	microlitre(s)
µm	micrometre(s)
ml	millilitre(s)
mM	millimolar
Na	sodium
NaCl	sodium chloride
NCCLS	national committe for clinical laboratory standards
ng	nanogram(s)
nm	nanometre(s)
nt	nucleotide(s)
O_2	oxygen
OD	optical density
O/N	overnight
%	percent
PCR	polymerase chain reaction
pmol	picomole(s)
QRDR	quinolone resistance determining region
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)

Ser	serine
spp.	species
subsp.	subspecies
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer
U	enzyme unit
UV	ultraviolet
V	voltage
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

ABSTRACT

Fluoroquinolone susceptible and resistant *Campylobacter coli* were isolated from pigs on two separate pig farms. *C. coli* are enteric pathogens of humans and animals and although diarrhoea resulting from *C. coli* and *C. jejuni* is generally a self-limiting disease, in severe cases, fluoroquinolones are the choice antibiotic for treatment. The presence of fluoroquinolone resistant *C. coli* strains in the food chain is cause for concern as this may be a source of resistant strains in humans.

Sixty-one isolates were included in the study: 26 were susceptible to nalidixic acid and ciprofloxacin and 35 were resistant to these antibiotics. Fifty-five strains were obtained from pigs on farm A, while 6 strains were obtained from pigs on farm B, the source farm of pigs to farm A. Serotyping and *flaA* typing were carried out to study the epidemiology of the isolates. Serotyping identified O:24 (11/61) as the most frequent serotype isolated, followed by O:5 (7/61). Common serotypes O:48, O:54 and O:59 were identified in strains from both farms. A high number of the strains were non-typeable (23/61) but were distinguished by *flaA* typing. RFLP analysis of the *flaA* gene revealed 13 distinct profiles in strains from farm A, and 4 profiles in strains from farm B, of which only 1 was unique to farm B. Profile 1 was the commonest profile observed with 31% (17/55) of *flaA* typed strains in this profile. There was an association between O:24, profile 6, and resistance. Resistant and sensitive pairs were isolated from 15 pigs; *flaA* profiles of each of 4 pairs were identical, suggesting selection of resistant mutants from previously sensitive populations.

An investigation of the molecular basis of the fluoroquinolone resistance identified a Thr-86 to Ile mutation in GyrA, the primary target of these antibiotics.

CHAPTER 1

LITERATURE REVIEW

1.1 THE GENUS *CAMPYLOBACTER*

The genus *Campylobacter* comprises a group of closely related curved or spiral, gram-negative bacteria that primarily colonise the gastrointestinal tracts of a variety of different host species (On, 1996). *Campylobacter* are non-spore forming rods, varying in length from 0.5 μm to 6.0 μm and a width of 0.2 μm to 0.5 μm. They are motile flagellate bacteria, possessing a single polar unsheathed flagellum at one or both ends (Fig 1.1), with a characteristic corkscrew-like, darting motion (Rollins & Colwell, 1986; Vandamme *et al.*, 1991). Cells of some species are nonmotile (*Campylobacter gracilis*) or have multiple flagella (*Campylobacter showae*).

Figure 1.1. An electron micrograph of *Campylobacter*. Original magnification, ×30,000. Kindly provided by Dr. L Stannard.

Campylobacter are micro-aerophilic and are damaged by normal atmospheric levels of O_2 (20%) and require O_2 levels below the range of 2-10%. Growth is achieved optimally at a temperature range of 30-42°C and high concentrations of carbon dioxide and hydrogen. These microorganisms are acid sensitive and do not grow below a pH of 4.9 making them fastidious and relatively slow growers. *Campylobacter* have a low G+C content ranging from 30-38% (Prescott *et al.*, 1996).

1.1.1 Taxonomy

The taxonomic classification of the genus *Campylobacter* has constantly evolved and has been extensively revised over the last century, particularly in the last 2 decades. Originally, the genus contained only two taxa, *Campylobacter fetus* and *C. bubulus* (Vandamme & Goosens, 1992; On, 1996). John McFadyean was the first to isolate campylobacters in pure culture from an aborted sheep foetus (McFadyean & Stockman, 1913). The "vibrio" like bacteria was called *Vibrio fetus*. In 1963, the genus *Campylobacter* was proposed to accommodate what had until then been regarded as microaerophilic vibrios and *Vibrio fetus* was changed to *Campylobacter fetus* (Vandamme & Goosens, 1992; Skirrow, 1994).

Since 1988, a new *Campylobacter* species has been identified every year. Currently 15 *Campylobacter* species and six subsp. are recognised. The main species making up the genus *Campylobacter* are: *C. fetus*, *C. hyointestinalis*, *C. concisus*, *C. mucosalis*, *C. sputorum*, *C. curvus*, *C. rectus*, *C. showae*, *C. gracilis*, *C. upsaliensis*, *C. helveticus*, *C. hyoilei*, *C. lari*, *C. jejuni* and *C. coli*. Most campylobacters and *Campylobacter*-like organisms have been assigned to rRNA superfamily VI that includes the genus *Helicobacter*, the family *Campylobacteraceae* and a number of other taxa (Vandamme *et al.*, 1991). Figure 1.2 summarises our present understanding of the taxonomy of the genus *Campylobacter*.

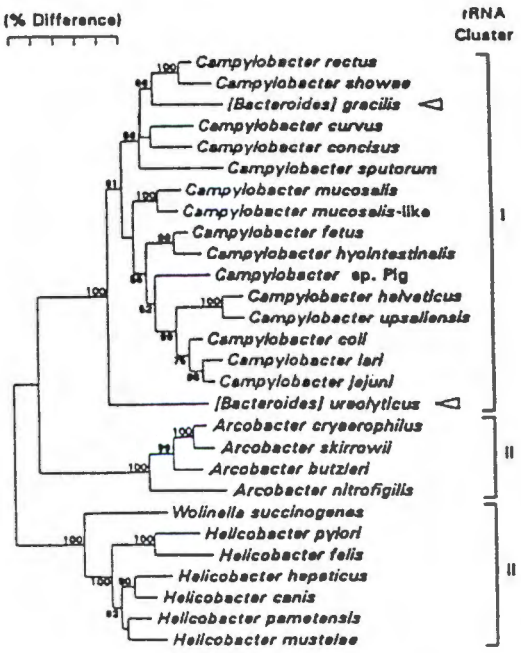


Figure 1.2. Phylogenetic tree of rRNA clusters within Superfamily VI (Vandamme *et al.*, 1995).

1.1.1.1 *C. jejuni & C. coli*

The two clinically important and most frequently studied species of *Campylobacter* are *C. jejuni* and *C. coli*, initially called *Vibrio jejuni* and *Vibrio coli* (King, 1962). *V. jejuni* was isolated from the faeces of cattle with diarrhoea (Laanbroek *et al.*, 1977; Kreig, 1984). Organisms similar to *V. jejuni* were also discovered in aborted sheep foetuses (Smibert, 1978). Subsequently *V. coli* was isolated from the faeces of pigs with diarrhoea (Vandamme & Goossens, 1992). *C. jejuni* and *C. coli* are enteropathogenic to humans and are recognised as one of the most frequent causes of acute diarrhoeal diseases in humans throughout the world. They, however, occur mostly as commensals in sheep, cattle, pigs, poultry, birds, various domestic animals, rodents, apes and even insects (Skirrow, 1977; Blaser *et al.*, 1980).

C. jejuni is divided into two subspecies, *C. jejuni* subspecies *jejuni and C. jejuni* subspecies *doylei* (Steele & Owen, 1988). *C. jejuni* subsp. *doylei* differs from *C. jejuni* subsp. *jejuni* biochemically by the absence of nitrate reduction and often catalase activity, although a weak reaction may be observed (Table 1.1). The taxonomic separation of *C. jejuni* subsp. *jejuni* and *C. coli* can be problematic. The phenotype and genotype of both taxa are remarkably similar. The most common and reliable test used to distinguish between the two is the hippurate hydrolysis test: with the exception of some strains of *C. jejuni subsp. jejuni*, *C. jejuni* is positive and *C. coli* is negative (Table 1.1) (Harvey, 1980). Further tests used to distinguish the two species include hydrogen sulfide production in triple-sugar-iron agar, growth on a minimal medium (On *et al.*, 1996) and utilization of propionate (Occhialini *et al.*, 1996).

Table 1.1 Differential diagnostic characteristics between *Campylobacter* species (Vandamme, 2000).

Campylobacter Species/subsp.	Alpha-hemolysis	Catalase	Hippurate hydrolysis	Urease	Nitrate reduction	Selenite reduction	H_2S/TSI (trace amounts)	Indoxyl acetate hydrolysis	Growth:							Resistance to:	
									25°C	42°C	Minimal medium	MacConkey	Glycine (1%)	NaCl (4%)	Cefoperazone (64 mg/lit)	Nalidixic acid	Cephalothin
C. jejuni subsp. *jejuni*	+	+	+	-	+	V	-	+	-	+	-	-	+	-	+	-	+
C. jejuni subsp. *doylei*	+	V	+	-	-	-	-	+	-	-	-	-	V	-	-	-	-
C. coli	V	+	-	-	+	+	V	+	-	+	+	V	+	-	+	-	+
C. concisus	V	-	-	-	V	V	-	-	-	V	-	-	V	-	-	V	-
C. curvus	V	-	V	-	+	-	V	V	-	V	V[a]	V[a]	+	-	V[a]	+	-
C. fetus subsp. *fetus*	-	+	-	-	+	V[a]	-	-	+	V[a]	V	V	+	-	+	+	-
C. fetus subsp. *venerealis*	V	V[a]	-	-	+	-	-	-	+	-	V[a]	V	-	-	-	V	-
C. gracilis	-	V	-	-	V[a]	-	-	V	-	V	V	V[a]	+	-	-	V	-
C. helveticus	+	-	-	-	+	-	-	+	-	+	-	-	V	-	V	-	-
C. hyointestinalis subsp. *hyointestinalis*	V	+	-	-	+	+	+	-	V	+	V	V	+	-	-	+	V
C. hyointestinalis subsp. *lawsonii*	V	+	-	+	+	+	+	-	-	+	V	V	V	-	V	+	-
C. lari	V	+	-	V	+	V	-	-	-	+	-	-	+	-	+	V	+
C. mucosalis	-	-	-	-	-	-	+	-	-	+	-	V[a]	V	-	V	V[a]	-
C. rectus	+	V	-	-	+	-	-	+	-	V	-	-	+	-	-	V[a]	-
C. showae	+	+	-	-	+	-	V	V	-	V	V	+	V	-	-	-	-
C. sputorum[b]	+	V	-	V	+	V	+	-	-	+	V	V	+	V	-	V	-
C. upsaliensis	+	-	-	-	+	+	-	+	-	V	-	-	+	-	V	-	V

[a] At least 80% of the strains examined contained this characteristic.
[b] Including bv. sputorum, faecalis, and paraureolyticus.
+ Characteristics present in over 90% of strains examined.
- Charcteristics present in less than 11% of the strains examined.
V Strain-dependent reaction.

1.1.2 Habitats and Ecology

Campylobacter spp. demonstrate considerable diversity in their ecology. They are ubiquitous in nature and can be isolated from anaerobic sludge, drinking water, seawater, birds, the genital and intestinal tracts of various animals and other sources (Table 1.2).

Table 1.2 Bacteria of the genus *Campylobacter* and their associations.
(Modified from Skirrow, 1994)

Species / subspecies	Main Host	Association
C. fetus subsp. *fetus*	Sheep, cattle	Ovine and bovine abortion; occasionally systemic infection in patients with immune deficiency
subsp. *venerealis*	Cattle	Infectious infertility in cows
C. hyointestinalis	Pig, cattle	Porcine proliferative enteropathy isolated occasionally from human patients with diarrhoea
C. mucosalis	Pig	Porcine proliferative enteropathy
C. sputorum subsp. *sputorum*	Man	Gingival flora
subsp. *bubulus*	Cattle	Genital flora, notably prepuce of bulls where it can be confused with *C. fetus* subsp *venerealis*
C. concisus	Man	Gingival crevices; peridontal Disease
C. curvus	Man	Gingival crevices; peridontal Disease
C. rectus	Man	Gingival crevices; peridontal Disease
C. showae	Man	Gingival crevices; peridontal Disease
C. jejuni subsp. *jejuni*	Birds, mammals	Acute enterocolitis in man and some animals
subsp *doylei*		Children with diarrhoea in developing world
C. coli	Pig, birds	Acute enterocolitis in man
C. lari	Birds, dog	Intestinal commensals; occasionally associated with diarrhoea in man
C. upsaliensis	Dog, cat	Dogs, cats and man, with and without diarrhoea; probably pathogenic for man
C. helveticus	Dog, cat	Dogs and cats, with and without diarrhoea

In cases of human infection, sources of contamination are mainly associated with milk and poultry products. *Campylobacter* have also been isolated from beef and pork. Campylobacters can survive in environments of temperatures as low as 4°C for several weeks and can also be present in surface water at higher temperatures. They have been found in natural water sources throughout the year. However, the presence of campylobacters does not clearly correlate with indicator organisms, such as *Escherichia coli*, for faecal contamination (Carter *et al.*, 1987).

As a result of this considerable diversity, many potential pathways of infection exist (Skirrow & Blaser, 1992). Generally the ecology of campylobacters focuses mainly on animal reservoirs where they reside in the intestinal tracts without causing clinical infection. Inanimate sources in the environment occur through contamination with animal faeces (NACMCF, 1993).

1.1.2.1 Campylobacter in Pigs and Pork

Studies carried out in the United States, Germany, Netherlands, Norway and Sweden have show that pigs are intestinal carriers of campylobacter species. Most campylobacter isolated from pigs are *C. coli*, however *C. jejuni* has also been isolated (Stern *et al.*, 1985; On, 1996). The presence of *Campylobacter* spp. in gallbladders and bile ducts of slaughtered pigs in Norway was described by Rosef (1981). He showed that 58% of the pigs sampled yielded *Campylobacter* spp. In another Nordic country, Sweden, Svedhem and Kaijser (1981) found that 95% of the pigs sampled at slaughterhouses yielded *Campylobacter* spp. A study carried out in the United States identified all campylobacter isolates from retail pork as *C. coli* (Stern *et al.*, 1985), and in the United Kingdom, a two year study revealed that 3.4% of campylobacter isolates from retail pork were identified as *C. jejuni* and 96.6% as *C. coli* (Fricker & Park, 1988). *C. jejuni* and *C. coli* are however, not the only *Campylobacter* species isolated from pigs. Other species including *C. hyointestinalis*, *C. mucosalis*, *C. sputorum* and *C. hyoilei* have been isolated from pigs but the incidence is less prevalent than is *C. jejuni* and *C. coli* (On, 1996). *C. hyointestinalis*, *C. mucosalis* and *C. hyoilei* are commonly isolated from proliferative mucosal lesions in pigs with proliferative enteritis (porcine intestinal adenomatosis, PIA) (Gebhart *et al.*, 1983).

1.2 CLINICAL SIGNIFICANCE

The clinical importance of *Campylobacter* to man was first suggested in 1946 by Levy who described an epidemic of gastroenteritis associated with the consumption of raw milk (Levy, 1946). In 1947, *V. fetus*, later changed to *C. fetus*, was isolated from a pregnant woman who had suffered a septic abortion (Blaser & Reller, 1981). It was in 1972, that clinical microbiologists first isolated campylobacters from stool samples of patients with diarrhoea (Kist, 1985). It was the selective media developed in the 1970s that allowed for more laboratories to test stool specimens for *Campylobacter* (Butzler *et al.*, 1973; Skirrow, 1977). Soon *Campylobacter* spp. were established as the common cause of human diarrhoea in most parts of the world (Allos & Blaser, 1995). In the United States, *C. jejuni* is now the leading cause of bacterial gastroenteritis (Tauxe, 1992). The Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Activity Surveillance Network reported in 1996 that 46% of laboratory-confirmed cases of bacterial gastroenteritis were caused by *Campylobacter* spp. followed by *Salmonella* (28%), *Shigella* (17%) and *E. coli* O157 (5%) (Figure 1.3). It is estimated that in the United States, there are between 2.1 to 2.4 million cases of human campylobacteriosis each year (Altekruse *et al.*, 1999).

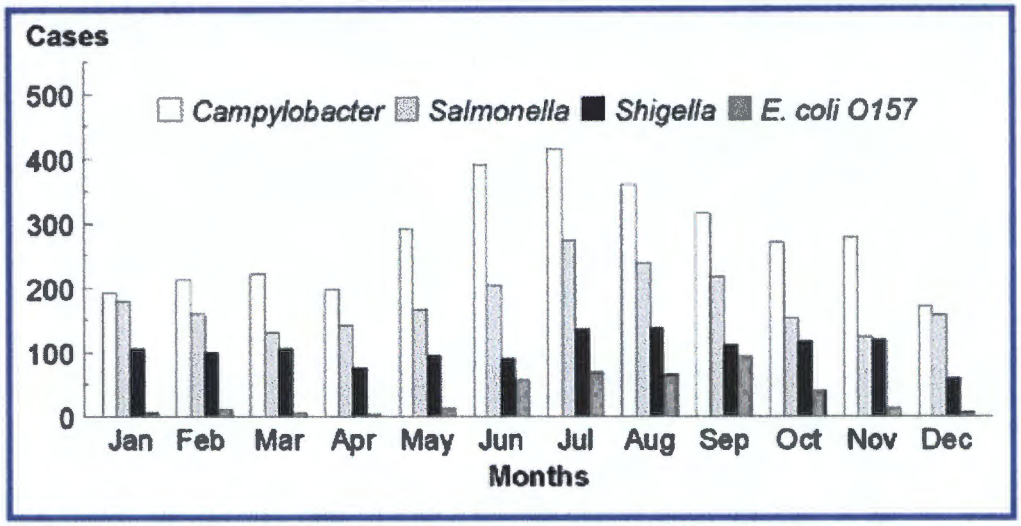


Figure 1.3. *Campylobacter* and other foodborne infections; Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Activity Surveillance Network, 1996 (Altekruse *et al.*, 1999).

C. jejuni subsp. *jejuni* and *C. coli* have been recognised since the late 1970s as important agents of gastrointestinal infections throughout the world and are currently the leading causes of bacterial gastroenteritis, worldwide (Tauxe, 1992). More than 95% of *Campylobacter* strains isolated and identified involved in cases of human disease have been *C. jejuni* subsp. *jejuni* or *C. coli* (Lastovica & Skirrow, 2000). However, of this 95%, *C. jejuni* accounts for 80-90% of infections, and 5-10% are due to *C. coli*.

Campylobacter infection, or campylobacteriosis, is an acute diarrhoeal disease with clinical manifestations similar to those of other acute bacterial infections of the intestinal tract. Clinically it cannot be distinguished from salmonellosis or shigellosis. A diagnosis can be made only by the detection of campylobacters in stool samples from patients. There does not seem to be any significant difference between infections caused by *C. jejuni* and *C. coli* (Skirrow & Blaser, 2000). Commonly, symptoms include diarrhoea, fever and abdominal cramping. Bloody diarrhoea is not uncommon in *Campylobacter* infection. Other symptoms often present are nausea, headache and muscular pain. The illness usually occurs 2-5 days after ingestion of contaminated food, mainly undercooked chicken, or water and generally lasts 7-10 days. Relapses are not uncommon and occur in about 25% of cases (U. S. Food & Drug Administration, 2000). Human feeding studies have shown that consumption of as little as 500 bacteria can cause illness. Host susceptibility can dictate infectious dose to some degree (Black *et al.*, 1988).

Campylobacter is one of the most common causes of foodborne disease in the United States. The vast majority of *Campylobacter* infections are not related to outbreaks but occur as sporadic individual infections. Common source outbreaks do occur which are normally small, consisting of less than 50 people. In Great Britain, campylobacteriosis is continually increasing. In 1998, 58,000 reported cases were recorded which far exceeds the reported cases of salmonellosis. Even though outbreaks due to *Campylobacter* spp. are rare, large-scale outbreaks have occurred such as one involving 2,500 children (Jones *et al.*, 1981) and one involving 2,000 people in a town using a non-chlorinated water source as a water supply (U. S. Food & Drug Administration, 2000). Infections are acquired through consumption of contaminated food; water or unpasteurised milk, however chicken is the single most important vehicle of transmission for sporadic cases in the United States and other developing countries due to improper handling and preparation of poultry (Tauxe, 1992).

Campylobacter infection plays an important role in the development of Guillian-Barré Syndrome (GBS). GBS, a neurological disorder resulting from demyelination of peripheral nerves can cause paralysis that leads to respiratory muscle failure and death (Nachamkin *et al.*, 1998; Allos, 1997a). GBS is the most common cause of acute neuromuscular paralysis, affecting 1 to 2 persons/100,000 population each year in many parts of the world (Blaser *et al.*, 1997). It is estimated that one case of GBS occurs for every 1,000 cases of campylobacteriosis and up to 40% of patients with GBS have evidence of recent *Campylobacter* infection (Allos, 1997b). Campylobacteriosis has also been associated with reactive arthritis, bacteraemia, pancreatitis, haemolytic ureamic syndrome, and following septicaemia, infection of nearly any organ. Table 1.3 summarises *Campylobacter* associated diseases in humans. Fatality due to campylobacteriosis is rare in healthy individuals, usually occurring only in cancer or immunocompromised patients. The estimated case/fatality ratio of all *C. jejuni* infections is 0.1, or one death per 1,000 cases (U. S. Food & Drug Administration, 2000).

Table 1.3 *Campylobacter* and associated human and animal diseases.

(Modified from On, 1996)

Species or subspecies	**Main source**	**Human disease Association**	**Animal disease Association**
C. jejuni subsp. *jejuni*	Birds, mammals	Gastroenteritis, septicaemia, Guillian-Barré syndrome	Gastroenteritis, avian hepatitis
C. jejuni subsp. *doylei*		Gastroenteritis, septicaemia, gastris	
C. fetus subsp. *fetus*	Sheep, cattle	Gastroenteritis, septicaemia	Spontaneous abortion in ovine & bovine
C. fetus subsp. *venerealis*	Cattle	Septicaemia	Bovine infectious infertility
C. coli	Pig, birds, bulls, sheep	Gastroenteritis, septicaemia	Gastroenteritis
C. lari	Birds, dogs, cats, monkeys, horses	Gastroenteritis, septicaemia	Avian gastroenteritis
C. hyointestinalis subsp. *hyointestinalis*	Pig, cattle, hamster	Gastroenteritis	Porcine and bovine enteritis
C. hyointestinalis subsp. *lawsonii*	Pigs		
C. concisus	Humans	Periodontal disease, gastroenteritis	
C. mucosalis	Pigs		Porcine enteritis
C. curvus	Humans	Periodontal disease, gastroenteritis	
C. rectus	Humans	Periodontal disease	
C. upsaliensis	Dogs, cats	Gastroenteritis, septicemia, abscesses	Canine and feline gastroenteritis
C. helveticus	Dogs, cats		Canine and feline gastroenteritis
C. showae	Humans	Periodontal disease	
C. sputorum bv. *sputorum*	Humans, cattle, pigs	Abscesses, gastroenteritis	
C. sputorum bv. *paraureolyticus*	Cattle		
C. sputorum bv *fecalis*	Cattle, sheep		
C. gracilis	Humans	Periodontal disease, deep tissue infection	

1.2.1 Pathogenesis

Although *Campylobacter* is recognised as one of the most important causes of human bacterial gastroenteritis, there is little knowledge of the pathogenesis of infection and controversy surrounds the number and specificity of toxic factors produced by these bacteria (Moutinho-Fragoso *et al.*, 1998). The pathogenesis of *Campylobacter* infection involves both host- and pathogen-specific factors. The age and health of the host and specific humoral immunity from previous exposure will influence clinical outcome after infection (Blaser *et al.*, 1987; Tauxe, 1992). There are many pathogen-specific virulence determinants that may play a role in pathogenesis of infection, however, none has a proven role. Major virulence factors contributing to pathogenicity include chemotaxis, motility, toxin production and invasive properties. Motility by means of flagella is the best-studied virulence factor and is considered to be a major factor, allowing the organism to penetrate the mucous layer above target epithelial cells (Ketley, 1997). Initially the jejunum and upper ileum are the sites of infection, followed by spread and infection to the rest of the ileum and colon. Acute inflammatory changes in the mucosa take place, often with crypt abscess formation. It is important for the bacteria to reach and attach to the mucosal surface. The spiral shape of campylobacters allows them to "corkscrew" their way through the thick mucous (Skirrow, 1994). Specific outer membrane proteins found in *C. jejuni* bind to epithelial cells (de Melo & Pechere, 1990), however, intact functioning flagella appear necessary for cell invasion (Grant *et al.*, 1993). Once colonisation occurs, other possible virulence determinants are iron acquisition, host cell invasion, toxin production, inflammation and active secretion, and epithelial disruption with leakage of serosal fluid (Ketley, 1997). Many strains produce cholera-like enterotoxins but in much smaller amounts than *Vibrio cholera*e or enterotoxigenic *Escherichia coli.* Cytopathic toxins have been detected in the supernatant fluids of *C. jejuni* cultures. The significance is however unclear (Skirrow, 1994). Bacterial invasion of epithelial cells ultimately results in cellular injury and consequently loss of cellular function and diarrhoea.

1.2.2 Treatment

The majority of patients with *Campylobacter* enteritis require no treatment other than the replacement of fluids and electrolytes lost through diarrhoea and vomiting. Severely dehydrated patients should receive rapid volume expansion with intravenous fluids. Most cases of *Campylobacter* enteritis do not require antimicrobial treatment

due to the infections being brief, clinically mild, and self-limiting (Dryden *et al.*, 1996). Antibiotic therapy is necessary for patients exhibiting high fever, bloody diarrhoea or more than eight stools in 24 hours; immunosuppressed patients, patients with bloodstream infections, and those with symptoms worsen or persist for more than 1 week from time of diagnosis. Antimicrobial therapy can reduce duration of illness, however delay in treatment may result in unsuccessful therapy.

Erythromycin was the first antimicrobial agent used in treatment of *Campylobacter* infection. Erythromycin binds to the ribosome and appears to cause dissociation of the peptidyl-tRNA, rather than blocking the peptidyltransferase activity (Prescott & Baggot, 1993). Ease of administration, lack of serious toxicity, and high degree of efficacy makes erythromycin the drug of choice for *C. jejuni* infection (Altekruse *et al.*, 1999). Resistance to erythromycin remains below 5% in most regions with exceptions such as Thailand where up to 50% of strains are resistant. *C. coli* isolates show a much higher level of erythromycin resistance than *C. jejuni*. Resistance to erythromycin in *C. jejuni* and *C. coli* is chromosomally mediated and is due to alteration of the ribosome (Taylor, 1992). Unfortunately cross-resistance exists between erythromycin and other macrolides (Skirrow & Blaser, 2000).

Other antimicrobial agents particularly the fluoroquinolones and newer macrolides including azithromycin are also used. Fluoroquinolones such as ciprofloxacin, were introduced in the 1980s and hailed with great enthusiasm, as they were effective against most major pathogen causing bacterial enteritis and offered a new approach to antibiotic intervention. Fluoroquinolones had good in vitro activity against all *Campylobacter* species as well as against members of the family *Enterobacteriaceae*, however, resistance to fluoroquinolones emerged in *Campylobacter* and this will be discussed in Chapter 3.

1.3 TYPING OF *CAMPYLOBACTER*

Subtyping of *Campylobacter* spp. is an important requirement for epidemiological studies, especially for (i) tracing sources and routes of transmission of human infections, (ii) identifying and monitoring temporally and geographically specific strains with important phenotypic characteristics, and (iii) developing strategies to control organisms within the food chain (Arbeit, 1995; Nicol & Wright, 1998; Newell *et al.*, 2000). Accurate identification and typing of *Campylobacter* spp. is also important from a clinical aspect. No standard subtyping technique has been

established, however, speed, cost, and ease of use are important factors to consider. A variety of methods have been used, each with its own unique advantage or disadvantage over other subtyping methods (Table 1.4).

Table 1.4 A comparison of genotyping methods and serotyping (Wassenaar & Newell, 2000).

Method	Discr. Power[1]	Typeability	Reprod[2]	Speed	Cost	Availability
fla typing	reasonable[3]	100%	good	<1 day	low	good
PFGE	good[4]	100%[5]	good	2-3 days	average	limited
ribotyping	poor	100%	good	3-4	average	complex method
-automated	good	NAV[6]	good	NAV	high	limited
RAPD	Average	80%	low	<1	low	good
AFLP	Good	100%	good	3-4	average	complex method
Sequencing	very good	100%	good	2-3	average	limited
Serotyping	Average	80%	good[7]	<1	low	limited

[1]Discriminatory power, is the ability to differentiate between genetically unrelated strains.

[2]Reproducibility of the method determines how duplicate samples give reproducible results. This is not dependent on external factors such as genetic instability.

[3]Horizontal exchange of flagellin sequences between strains may influence the discriminatory power of the method, however incidence that such exchange can take place outside the laboratory is limited to date.

[4]Incidentally genomic instability is detected by PFGE which could lead to false interpretation, however the frequency of such events is unknown.

[5]The sensitivity of PFGE is lower than 100% for DNAse producing strains, but this can be overcome by experimental modifications.

[6]Not available.

[7]Weak serological cross-reactions can complicate the interpretation. Multiple passages may be required to fully express antigens.

For epidemiological purposes the most widely used phenotypic procedure has been serotyping but this method usually lacks adequate discriminatory power. Many DNA based (genotypic) subtyping schemes have been developed including pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) analysis, flagellin gene typing and ribotyping. Table 1.4 shows that most genotyping techniques have a higher typeability than serotyping. Often combinations of serotyping and genotypic techniques are used resulting in higher discriminatory power and therefore a more complete strain identification (Wassenaar & Newell, 2000).

1.3.1 Serotyping

In the 1980s, two serological subtyping methods were developed. The Penner scheme is based on soluble heat-stable (HS) or somatic O antigen (Penner & Hennessey, 1980) and the Lior scheme is based on variation in the heat-labile (HL) antigens (Lior *et al.*, 1982). Currently there are 108 HL serotypes based on the bacterial agglutination technique of Lior *et al.*, (1982) that are used to type *C. jejuni* and *C. coli*. The serotyping scheme by Penner and Hennessey identifies 47 heat stable serotypes of *C. jejuni* and 19 serotypes of *C. coli*.

The Penner scheme has been more extensively used and forms the basis of the scheme developed in the Laboratory of Enteric Pathogens (LEP), Public Health Laboratory Services, United Kingdom. The LEP scheme now forms the basis of reference typing in the UK and was evaluated by using 2,407 *C. jejuni* and 182 *C. coli* isolates. Forty-seven serotypes were identified for *C. jejuni* and 15 serotypes were identified for *C. coli*. Non-typeability is a shortcoming of serotypic schemes. Using the LEP serotyping scheme, 19% of human isolates were non-typeable (Frost *et al.*, 1998), with up to 63% of human isolates utilising the Penner scheme being non-typeable from some countries (Asrat *et al*, 1997). Further disadvantages of serotyping include: it is labour intensive, time consuming and antisera required for typing are not widely available, limiting serotyping almost exclusively to reference laboratories. Despite these shortcomings, the Penner scheme of serotyping has been important in linking particular serotypes with GBS (Kuroki *et al.*, 1993; Goddard *et al.*, 1997; Lastovica *et al.*, 1997). Kuroki *et al.*, (1993) confirmed the association between serotype O:19 *Campylobacter* isolates and GBS. Similarly, research by Yuki *et al.* (1997) also found that O:19 strains were associated with GBS and that serotype O:2 was overexpressed in patients with Miller-Fisher syndrome (MFS), another neurological disorder. In South Africa, Goddard *et al.*, (1997) and Lastovica *et al.*, (1997) identified an additional serotype, O:41, in patients with GBS.

1.3.2 Molecular Typing

There is a well-recognised need for alternative subtyping schemes due to the limitations of serotyping. Only recently have molecular subtyping techniques been developed to address the shortcomings of phenotypic methods (Wassenaar & Newell, 2000). Major advantages of genetically based methods are their enhanced sensitivity and discrimination. Genotyping methods have proved their worth as typing tools and are potentially available worldwide (Wassenaar & Newell, 2000). Genotypic techniques, such as pulsed field gel electrophoresis (PFGE), ribotyping and flagellin gene typing are currently being applied to epidemiological studies, especially from veterinary sources (Newell & On, 1998; Newell *et al.*, 2000).

1.3.2.1 Pulsed field gel electrophoresis

PFGE is considered to be one of the most powerful tools available for microbial molecular epidemiology. The technique is highly sensitive in that whole genome restriction site polymorphisms are detected. The technique was described initially for *C. jejuni* (Yan *et al.*, 1991) and was later adapted for *C. coli*, *C. hyointestinalis*, *C. fetus* and *C. upsaliensis* (Salama *et al.*, 1992; Fujita *et al.*, 1995; Bourke *et al.*, 1996).

PFGE requires bacterial cells to be embedded in agarose and lysed in situ to prevent DNA shearing, which would introduce random breaks. Extensive washing is carried out to remove contaminating chemicals such as formaldehyde that are used in cases to deactivate DNase activity of some thermophilic *Campylobacter* strains. The intact DNA contained in an agarose slice is cleaved with an endonuclease and the restriction site polymorphisms are detected by PFGE. Restriction enzymes cleave the DNA infrequently, resulting in a few large fragments (20-200 kb). Restriction enzymes used include *Sma*I, *Sal*I and *Kpn*I for genotyping *Campylobacter* spp. The various DNA fragments obtained are separated by special electrophoretic conditions. Pulse electric fields are applied from different positions in the electrophoresis cell.

Even though PFGE has a higher discriminatory power then other typing techniques, there are a number of disadvantages with PFGE: (i) the apparatus required for electrophoresis is specialised and expensive; (ii) preparation of the DNA-containing agarose blocks is tedious and time consuming; (iii) considerable variation in restriction enzymes and electrophoretic conditions between laboratories makes comparison of PFGE profiles difficult; (iv) genetic instability can lead to minor or major changes in profiles; (v) degradation of DNA due to DNase of some strains; and (vi) the DNA of different strains is undigestable with the enzymes used for PFGE.

1.3.2.2 Ribotyping

Ribotyping makes use of the presence of multiple copies of the ribosomal RNA genes coding for 16S and 23S rRNA at different positions in the chromosome. These genes are suitable for targeting for subtyping purposes due to the strong conservation of regions within the rRNA genes between bacteria, and the presence of highly variable, non-coding, flanking regions. The most commonly used technique is Southern blot hybridisation of restricted DNA, hybridised with a probe specific for rRNA genes. The majority of researchers use probes derived by PCR from *Campylobacter* DNA and specific for 16S rRNA (Wassenaar & Newell, 2000). Most *Campylobacter* spp. have three ribosomal gene copies, fewer than other bacteria which reduces the discriminatory power. Generally, ribotyping is best suited for speciation of *Campylobacter* isolates difficult to identify phenotypically (Kiehlbauch *et al.*, 1991), although the technique has successfully been applied to differentiate between *C. jejuni* in an outbreak situation (Tee *et al.*, 1992). Subtyping of *C. coli* (Stanley *et al.*, 1995), *C. upsaliensis* (Goossens *et al.*, 1995), *C. lari* (Owen *et al.*, 1993a) and *C. helveticus* (Kiehlbauch *et al.*, 1991) has also been achieved.

Ribotyping is unsuited as a routine genotyping procedure due to its low discriminatory power and it is a laborious, time-consuming technique. Development of a completely automated ribotyping procedure for identification and characterisation of bacteria enhances reproducibility and saves time. However, the high costs of equipment and consumables limits the use of this technology.

1.3.2.3 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) is a PCR based fingerprinting technique (Welsh & McClelland, 1990; Williams *et al.*, 1990). Arbitrarily designed primers are used for the amplification of random DNA products under low-stringency conditions. The method uses the entire genome of the target organism to generate amplified fragments. The number and size of the fragments generated are partially controlled by stringency regulation through manipulations of the annealing temperature. RAPD does not require prior knowledge of the target DNA sequence unlike other PCR based typing methods, such as flagellin gene typing. Amplification varies due to the length of the product formed and the efficiency of annealing, resulting in banding patterns consisting of stronger and weaker amplicons of variable length. Banding patterns obtained are suitable for comparison of isolates within a

bacterial species. RAPD has been applied to typing human, animal and environmental isolates of *C. jejuni*, *C. coli* and *C. lari* (Madden *et al.*, 1996). A slight variation to the amplification of random genomic DNA fragments is the use of primers specific for enterobacterial repetitive intergenic consensus sequences (ERIC) (Sharples & Lloyd, 1990). ERIC primers can be used under high stringency to match the target sequences. This method has been used to genotype *C. jejuni*, *C. upsalienesis* (Giesendorf *et al.*, 1994) and *C. coli* in pigs (Weijtens *et al.*, 1999).

RAPD has the advantage of being much quicker and cheaper than PFGE and does not require complex apparatus. However, problems have been associated with reproducibility. Meunier and Grimont, (1993) noted that the source of *Taq* DNA polymerase and type of thermocycler could significantly affect the patterns obtained. Standardised conditions are now more readily available following improvements in thermocylers and commercially prepared reagents (Newell *et al.*, 2000).

1.3.2.4 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) was originally developed for the genetic analysis of plants (Vos *et al.*, 1995) and has been adapted for genotyping of bacteria (Lin & Kuo, 1995; Janssen *et al.*, 1996). AFLP has proven to be a useful typing tool for *Campylobacter* (Duim *et al.*, 1999; Kokotovic & On, 1999).

The method involves chromosomal DNA digestion with two restriction enzymes. The enzymes of choice are dependant on the G+C content and the size of the genomic DNA. Amplification by PCR of the digested products is based on the restriction sites and designed such that only those fragments flanked by both restriction sites are amplified. The primers are either radioactively labelled (Vos *et al.*, 1995) or flourescently labelled (Kokotovic & On, 1999). The labelled PCR products are analysed on denaturating polyacrylamide gels. Optimally 80-100 bands are generated which can be reduced by incorporation in the PCR primers, of one or more specific nucleotides adjacent to the restriction site. Therefore only fragments with specific nucleotides adjacent to restriction site will be detected and analysed (Wassenaar & Newell, 2000) on an automated DNA seqeuncer.

AFLP has been successfully used for the differentiation between isolates of *H. pylori* and *Campylobacter* (Gibson *et al.*, 1998; Kokotovic & On, 1999) and has recently been developed for subtyping *C. jejuni*. AFLP has also been successfully used to investigate the epidemiology of poultry *Campylobacter* (Duim *et al.*, 1999). An

advantage of this technique is that single nucleotides can be resolved. AFLP is rapid and easily standardised, however the equipment is expensive. With the digitisation of AFLP results, accurate interpretation, ease of data storage and data exchange between research institutes is possible.

1.3.2.5 Flagellin gene typing

Flagellin gene typing (*fla* typing) was developed by Nachamkin *et al.*, (1993) for sub-typing *C. jejuni* (Nachamkin *et al.*, 1993); subsequently the technique has been applied to typing other *Campylobacter* spp. (Owen *et al.*, 1993b). Flagellin is the major protein subunit of the flagellar filament involved in motility. The flagellin gene locus of *C. jejuni* comprises two flagellin genes, *fla*A and *fla*B, which are arranged in tandem repeats and separated by a 170 bp intergenic region. These genes are highly conserved, with 95% identity between *fla*A and *fla*B genes in individual isolates (Nuitjten *et al.*, 1990). Due to both highly conserved and variable regions that are present (Meinersmann *et al.*, 1997), the flagellin gene locus is suitable for restriction fragment length polymorphism (RFLP) analysis of a PCR product. PCR targets either the *fla*A or both the *flaA* and *flaB* genes for amplification by use of conserved primers. The amplicons are digested with restriction enzymes resulting in fragments that can be analysed by agarose gel electrophoresis (Ayling *et al.*, 1996; Nachamkin *et al.*, 1993; Nachamkin *et al.*, 1996). Variations between strains will be reflected by differences in enzyme recognition sites present in less conserved regions of the PCR product.

At least 7 *fla* typing methods that have been developed (Alm *et al.*, 1993a; Ayling *et al.*, 1996; Birkenhead *et al.*, 1993; Burnens *et al.*, 1995 King & Clayton, 1991; Nachamkin *et al.*, 1993; Nishimura *et al.*, 1996) with considerable differences in the PCR/RFLP method (Table 1.5). Differences include DNA preparation, primer design, restriction enzymes used, annealing temperature and nomenclature of genotype. Differences between the primers can result in significant variation in the profiles obtained from the same strains. The majority of the primer sets have been designed to amplify *fla*A specifically (Burnens *et al.*, 1995; Nachamkin *et al.*, 1993; Nishimura *et al.*, 1996), however some methods make use of amplification of both the *fla*A and *fla*B using combinations of three primers (Alm *et al.*, 1993a; Ayling *et al.*, 1996; King & Clayton, 1991) (Table 1.5).

Table 1.5 A summary of flagellin gene typing methods for *Campylobacter*.
(Modified from Matsheka, 2000)

Reference	Species typed	Primers		PCR product and size	Restriction enzyme
		Forward 5'→ 3'	Reverse 5'→ 3'		
Alm *et al.*, (1993a)	*C. jejuni* *C. coli*	5' ATGGGATTTCGTATTAAC 3' 5' AAGGATTTAAAATGGGTT TTAGAATAAACACC 3'	5' GCACC[CT]TTAAG[AT] GT[AG]GTTACACCTGC 3'	*fla*A and *fla*B 1448bp	*Pst*I, *Eco*RI
Ayling *et al.*, (1996)	*C. jejuni*	5' AAAGGATCCGCGTATTAA CACACAAATGTTGCAGC 3' 5' AAAGGATCCGAGGATAAA CACCAACATCGGT 3'	5' GATTTGTTATAGCAGTT TCTGCTATATCC 3'	*fla*A and *fla*B 1490bp	*Dde*I, *Alu*I, *Hin*fI
Burnens *et al.*, (1995)	*C. jejuni* *C. coli*	5' ccggatcccATGGCATTTCGTA TT 3'	5' ttcgaattCTATTGTAATAA TCTTAAAACAT 3'	*fla*A 1731bp	*Alu*I, *Dde*I, *Hae*III, *Hin*fI
King & Clayton, (1991)	*C. jejuni*	5' ATGGGATTTCGTATTAAC AC 3'	5' CTATTGTAATAATCTTA AAA 3'	*fla*A and *fla*B 1723bp	*Hae*III, *Bgl*II
Nachamkin *et al.*, (1993, 1996)	*C. jejuni* *C. coli*	5' GGATTTCGTATTAACACA AATGGTGC 3'	5' CTGTAGTAATCTTAAAA CATTTTG 3'	*fla*A 1728bp	*Dde*I
Nishimura *et al.*, (1996)	*C. jejuni*	5' TACTACAGGAGTTGAGCT T 3'	5' GTTGATGTAACTTGATT TTG 3'	*fla*A 702bp	*Hae*III, *Afa*I, *Mbo*I

Regions of DNA homology are indicated in red (forward primers) and blue (reverse primers). Sequences utilised for cloning shown by lower case nucleotide bases.

As a result of the high conservation between *fla*A and *fla*B, addition of a *fla*B specific primer only contributes to differentiation in less than 10% of isolates (Alm *et al.*, 1993b; Mohran *et al.*, 1996). Most of the forward primers designed match the start of the *flaA* gene with slight variation between methods. The reverse primers however vary resulting in different sized PCR products (Table 1.5). Choice of restriction enzyme varies significantly between studies. The enzymes currently in use include *Dde*I, *Hinf*I, *Alu*I, *EcoR*I and *Pst*I. Ayling *et al.*, (1996) report that the enzyme *Dde*I provides the best discriminatory power that can be enhanced by use of *Hinf*I in combination.

*fla*A typing is a useful tool for subtyping clinical and epidemiological investigations (Nachamkin *et al.*, 1993). It is quick, simple and has a high level of discrimination. The technique has been applied to *Campylobacter* from broiler flocks, foodstuffs and from animals and water (Ayling *et al.*, 1996; Lorenz *et al.*, 1998). The method has also been applied as an epidemiological tool to investigate human isolates clustered by Penner serotyping (Slater & Owen, 1998) as well as strains associated with GBS (Fujimoto *et al.*, 1997). However there are disadvantages with this method. The choice of primer sets, combined with the variation of PCR parameters make comparison of results between research institutes difficult unless standardisation of the flagellin typing technique occurs.

1.4 AIMS OF THE STUDY

Recently, fluoroquinolone resistant *Campylobacter coli* were isolated from a pig farm in Stellenbosch, South Africa. Fluoroquinolones were introduced into veterinary medicine in South Africa approximately 6 years ago. Since then there has been a steady increase in resistant isolates. *C. coli* is the most frequently isolated species of *Campylobacter* from pigs. The emergence of porcine fluoroquinolone resistant *C. coli* isolates is cause for concern as pigs are a food source and may be a source of resistant strains in humans.

Therefore the aims of this project are two fold:

1). To study the epidemiology of sensitive and resistant isolates using serotyping and molecular typing.

2). To determine the molecular basis of fluoroquinolone resistance in *C. coli*.

CHAPTER 2

PHENOTYPING & GENOTYPING OF *CAMPYLOBACTER COLI* ISOLATES

2.1 INTRODUCTION

C. coli and C. jejuni are considered to be among the most important enteric pathogens worldwide. While *C. jejuni* is commonly isolated from chickens, *C. coli* is the dominant species isolated from pigs. Campylobacters were first described in pigs in 1944 by Doyle, who thought they were the cause of swine dysentery (Doyle, 1944). He thought the organism was a vibrio and so proposed the name "*Vibrio coli*", which was subsequently changed to *C. coli* (Laanbroek *et al.*, 1977; Kreig, 1984). The principal agent of swine dysentery is in fact *Serpulina* (*Treponema*) *hyodysenteriae,* although there is evidence that anaerobic bowel flora and *C. coli* may play a role in pathogenesis of the disease (Fernie *et al.*, 1975). There has been debate over whether *C. coli* alone is enteropathogenic for pigs with reports of its presence in large numbers and as the sole putative pathogen in the intestines of pigs with diarrhoea, however it is also found in most healthy pigs (Skirrow, 1994).

Numerous epidemiological reports describe food of animal origins as sources of campylobacteriosis in humans. Poultry is the main link with *Campylobacter* infection and has often been associated with outbreaks of campylobacteriosis (Skirrow *et al.*, 1981). In contrast, pork has been implicated in a limited number of outbreaks (Yanagisawa, 1980). Although pigs are a natural reservoir of campylobacters, not much is known about their epidemiology in these animals. On the other hand, many epidemiological studies have been carried out on *C. jejuni* in chickens.

In the recent past, serotyping has been used to study the epidemiology of *Campylobacter*. The two schemes used for epidemiological studies are based on heat-stable antigens (Penner & Hennessey, 1980), and heat-labile antigens (Lior *et al.*, 1982). These methods have proved valuable and have tended to be the "gold standard" for typing (Wassenaar & Newell, 2000); however, they are limited as not all isolates can be serotyped, and specific antisera are of limited availability. Furthermore, isolates of the same serotype may be heterogenous and only distinguishable by additional typing methods (Owen *et al.*, 1995). Due to such problems, the need for an alternative subtyping technique was recognised. In 1993, Nachamkin *et al.*, (1993) developed a molecular typing technique for *C. jejuni* utilising restriction fragment length polymorphism (RFLP) of the *flaA* gene. The results suggested that the method was sufficiently discriminatory and could provide an alternative to serotyping as a typing method for clinical and epidemiological investigations (Nachamkin *et al.*, 1993). Ayling *et al.* (1996) developed a similar PCR approach that they applied to veterinary epidemiological investigations for subtyping

C. jejuni isolates. The method was validated by using strains from 28 serotypes of *C. jejuni* and was stated to be suitable for typing *C. coli* (Ayling *et al.*, 1996). The method was optimised for isolates of *C. jejuni* and was used to investigate campylobacters from chickens in 15 broiler flocks. The potential value of this technique as a typing tool for veterinary epidemiology investigations was determined by comparing it with the Penner serotyping scheme. All the isolates could be subtyped using the RFLP method and no direct relationship between serotype and RFLP was observed. The RFLP/PCR typing method utilising the flagellin genes provides a suitable tool for the investigation of the routes of transmission and sources of campylobacters in chickens (Ayling *et al.*, 1996). Burnens *et al.*, (1995) looked at the relationship between Lior heat-labile serogroups of *C. jejuni* and *C. coli* and the RFLP of the flagellar genes to determine if any possible correlation existed. Limited correlation between flagellar RFLP and the Lior serogroup existed (among 85 seroreference strains, 74 different RFLP patterns were observed) with flagellar gene RFLP providing a marker that was highly discriminatory between serotypes (Burnens *et al.*, 1995). Based on the above and the observations of others as shown in Table 1.4, *flaA* typing has been used successfully to type *Campylobacter* spp. including *C. coli*.

This chapter describes the typing of porcine isolates of *C. coli* using the Penner scheme of serotyping in conjunction with the molecular technique, *flaA* typing.

2.2 EXPERIMENTAL PROTOCOL

2.2.1 Bacterial Isolates and Susceptibility Testing

Rectal swabs were taken from pigs at a pig farm in Stellenbosch, South Africa, in October and December, 1999. Campylobacters were isolated from the swabs taken by Dr. T. Gous of the Regional Veterinary Laboratories Stellenbosch. Isolates were identified as *C. coli* by E. Le Roux using morphological and biochemical characteristics consistent with the genus *Campylobacter*. Sixty one isolates were included in the study. A reference strain, *C. coli* NCTC 11283, was kindly provided by Dr. A. Lastovica, Red Cross Children's Hospital, Cape Town and the Department of Medical Microbiology, University of Cape Town, Cape Town. Disc susceptibility testing using nalidixic acid (30 μg) and ciprofloxacin (5 μg) was carried out according to the National Committee for clinical Laboratory Standards criteria (National Committee for Clinical Laboratory Standards criteria, 1998a).

2.2.1.1 Culture Maintenance

C. coli samples were plated onto tryptose blood agar (TBA) plates containing 10% unlysed horse blood. Cultures were incubated for 2 days at 37°C in a H_2-enhanced microaerophilic atmosphere generated by use of Oxoid BR 38 gas generating kit.

Short term storage (less than 7 days) plates were stored under microaerophilic conditions at 37°C or stored at 4°C.

Long term storage, bacteria were scraped off the TBA plates and resuspended in glycerol broth (1ml of 30% v/v glycerol, 70% 2× YT broth (Appendix A)) and stored at -70°C.

2.2.2 Preparation of Genomic DNA

Two methods were used to isolate DNA.

2.2.2.1 Genomic DNA Extraction Kit Method

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega). *Campylobacter* cells were harvested from TBA plates and gently resuspended in 600 μl of Nuclei Lysis Solution (Promega). The resuspension was

incubated at 80°C for 5 minutes allowing for lysis of the cells. Contaminating RNA was removed by the addition of 3 µl of RNase solution (200 µg/ml), at 37°C for 1 hour. The sample was cooled to room temperature (RT), 200 µl of Protein Precipitation Solution (Promega) was added, vortexed and the sample was placed on ice for 5 minutes; after which it was centrifuged (Eppendorf 5417C benchtop centrifuge) at 14,000 rpm for 4 minutes to remove cellular protein. The clear lysate was removed and the DNA was recovered, following the addition of isopropanol (600 µl), by centrifugation at 14,000 rpm for 2 minutes. The DNA pellet was washed with 70% ethanol and allowed to dry for 10-15 minutes. The DNA pellet was rehydrated by the addition of 100µl of DNA Rehydration Solution (10 mM Tris-HCl, 1 mM EDTA) and incubated for 1 hour at 65°C to facilitate the process. The DNA solution was stored at –20 °C.

2.2.2.2 Guanidium Thiocyanate DNA Extraction

Genomic DNA was extracted by use of guanidium thiocyanate method (Pitcher *et al.*, 1989). *Campylobacter* cells were harvested and resuspended in 100 µl of Tris-EDTA (TE) buffer (Appendix A) and lysed with 500 µl of guanidium thiocyanate solution (Appendix A). The solution was vortexed briefly and placed on ice when lysis was evident. Ice cold (250 µl) 7.5M ammonium acetate (Appendix A) was added to the solution, mixed and left on ice for 10 minutes. Five hundred microlitres of chloroform-isoamylalcohol (Appendix A) was added and the solution was centrifuged (Eppendorf 5417C benchtop centrifuge) at 10,000 g for 10 minutes. The supernatant was added to a 0.54 volume of ice-cold isopropanol, mixed and briefly centrifuged to collect the DNA. The pellet was washed 3 times with 70% ethanol to remove traces of guanidium thiocyanate. The ethanol was drained and the pellet air-dried and resuspended in 100 µl of TE buffer.

2.2.3 DNA Concentration Determination

Genomic DNA concentration was determined by agarose gel electrophoresis and by UV spectrophotometry. Aliquots of sample DNA were electrophoresed next to titrated concentrations of lambda-DNA (λ-DNA) (Promega) commencing with 50 ng. DNA concentration was estimated by comparison with λ-DNA of known concentrations.

UV spectrophotometry was used to determine DNA concentration of genomic DNA. The DNA was diluted 100-fold and the DNA concentration was determined by

measuring the absorbance at 260 nm in a Hewlett Packard Diode Array Spectrophotometer using the HP 8452 Win System and the 1 unit to 50 mg/ml relationship. DNA was diluted to approximately 200 ng/µl for use in later applications.

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in submerged 0.8-1.5% agarose gels (Techcomp, Ltd.) dissolved in 1× Tris-acetate (TAE) buffer (Appendix A). The migration rate of DNA is dependent on the molecular size of the DNA, the agarose concentration and the DNA conformation (Maniatis *et al.*, 1982). Negatively charged DNA migrates from the anode to the cathode when an electric field is applied across the gel. To visualise the DNA, ethidium bromide (EtBr) (Appendix A) was added to the agarose solution at a concentration of 20 ng/ml and to monitor migration, sample DNA was mixed with tracking dye (2 µl) (Appendix A). Samples were loaded into wells and electrophoresed next to a molecular weight marker, 1kb DNA ladder (Promega) (Appendix B). DNA was visualised under an ultra violet light source (302 nm) and photographed using a Kodak Digital DC 120 camera.

2.2.5 Serotyping

Serotyping of the 61 pig isolates was carried out with Dr. Lastovica on the basis of the thermostable somatic (O) lipopolysaccharide antigens performed with antisera to the 66 serotype reference strains of *C. jejuni* and *C. coli* by the scheme of Penner & Hennessy (1980). Isolates were also tested against an additional 12 antisera to new serotypes not included in the scheme (Le Roux & Lastovica, 1998).

The passive haemagglutination technique was used for titration of antisera. For antisera titration with saline extracted or EDTA-extracted antigens, extracts were diluted 1:10 in phosphate-buffered saline (PBS) (Appendix A). Diluted extracts were incubated at 37°C for 1 hour with an equal volume of a 1% suspension of sheep erythrocytes washed previously in PBS. Centrifugation of the sensitised erythrocytes was followed by three washes in PBS and cells were resuspended in PBS to a final concentration of 0.5%. Two fold dilutions were prepared in a microtitration plate with wells containing 0.025 ml of PBS. To each well of diluted antisera, 0.025 ml of

sensitised erythrocytes was added. Plates were shaken and incubated for 1 hour at 37°C and stored at 4°C overnight. Plates were read by examining for agglutination of erythrocytes. The highest dilution of antiserum exhibiting agglutination was taken as the titre. Antisera were initially diluted 1:40 and absence of agglutination at this dilution was taken as a negative reaction (Penner & Hennessy, 1980).

2.2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify DNA sequences of interest. Amplification occurs in the presence of dNTPs, 1× PCR buffer, *Taq* polymerase and two oligonucleotide primers. Primers are designed to be complementary to sequences flanking the target DNA segment. PCR reaction involves the denaturation of the original double-stranded DNA sample at high temperature, followed by annealing of the primers to the DNA template at an appropriate temperature. Extension of the primers occurs by use of a thermostable DNA polymerase, *Taq* polymerase. These steps are repeated in cycles resulting in the exponential synthesis of target DNA (Fig 2.1). (Saiki *et al.*, 1988).

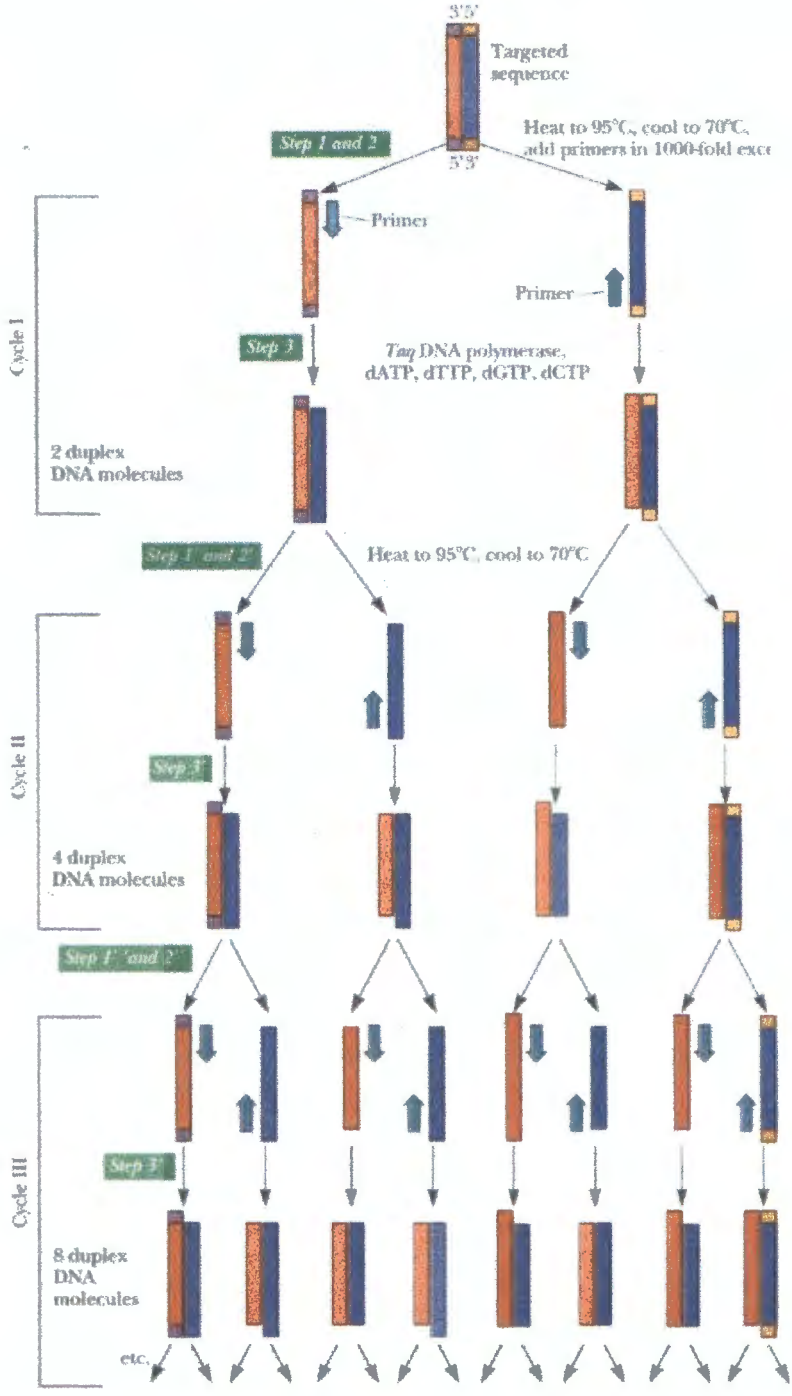


Figure 2.1 Schematic diagram of polymerase chain reaction (PCR) (Garrett & Grisham, 1995).

2.2.6.1 16S rRNA Gene Amplification

To determine the suitability of template DNA for PCR, the 16S rDNA was amplified using primers designed to amplify a 1,004 bp fragment within the coding region of the 16S rRNA gene in *Campylobacter*, *Arcobacter* and *Helicobacter* (Marshall *et al.*, 1999). The forward and reverse primers used were CAH 16S 1a (5'-AATACATGCAAGTCGAACGA-3') and CAH 16S 1b (5'-TTAACCCAACATCTCACGAC-3'), respectively. Primers were synthesized by GIBCO-BRL (Life Technologies). The 16S PCR amplification method was performed in a 50 µl reaction volume containing approximately 200 ng DNA, 0.5 µM each primer, 1× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin (TaKaRa), 200 µM of each dNTP (TaKaRa), 2.5 units *Taq* polymerase (TaKaRa). PCR amplification was performed in a Perkin Elmer GeneAmp PCR System 2400. Initial denaturation was carried out at 95°C for 120 seconds, followed by 30 amplification cycles, each consisting of 94°C for 60 seconds, 52°C for 45 seconds and 72°C for 120 seconds. A final primer extension at 72°C for 10 minutes was included. Aliquots (10 µl) of PCR products were electrophoresed on a 1.5% agarose gel at 100V for 1 ½ hours.

2.2.7 Molecular Typing

2.2.7.1 Flagellin Gene (*flaA*) Typing

A number of sets of primers were used to amplify the *flaA* gene of *C. jejuni* and *C. coli* (Table 2.1). Figure 2.2 is a schematic diagram of the *flaA* gene showing primer binding sites.

Table 2.1 Primers used in the amplification of *flaA*.

Primer	Orientation (Forward/Reverse)	Sequence (5'→3')	Reference
Cj 431	Forward	AAAGGATCCGCGTATTAACACAAATGTTGCAGC	Ayling *et al.* (1996)
Cj 433	Reverse	GATTTGTTATAGCAGTTTCTGCTATATCC	Ayling *et al.*, (1996)
Cc 431	Forward	TTCGTATTAACACAAATGTTGC	This study
Cc 433	Reverse	CGTGCGCTAGATACTATCTG	This study
A2	Reverse	CTGTAGTAATCTTAAAACATTTTG	Nachamkin *et al.*, (1993)

Figure 2.2 A schematic representation of the *flaA* primer binding sites. The primers (arrows) and their target sites (bases) are shown. Primers are not drawn to scale.

PCR amplifications were performed in solutions containing 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin (TaKaRa); 2.5 mM of each dNTP (TaKaRa); 20 pmol of each primer; 2.5 units *Taq* polymerase (TaKaRa); 200 ng of genomic DNA; and ultrapure water made up to a final volume of 50 µl. A control solution was used in which ultrapure water was used in place of genomic DNA. The initial denaturation was at 94°C for 60 seconds, followed by 45 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 120 seconds. A final extension took place at 72°C for 5 minutes. Aliquots of the PCR products were gel (1.5%) electrophoresed (100V for 1 ½hr) and visualised by UV transillumination.

2.2.7.2 RFLP Analysis

Restriction digestions (20 µl) containing an aliquot (8 µl) of the PCR products was digested with *Dde*I (10 units) (GibcoBRL®, Life Technologies) at 37°C overnight in the buffer reaction conditions specified by the manufacturer. The enzymatic reaction was stopped by the addition of 6× tracking dye (Appendix A). Following digestion, he fragments were separated in a 2.5% agarose gel at 30V for 16 hours.

2.3 RESULTS

2.3.1 *Campylobacter* Isolates

The 61 porcine isolates used in this study are shown in Table 2.2: 55 of the strains (2R-73R) came from pigs on farm A and were isolated in October 1999. The remaining isolates (2MF2034-10MF2039) were obtained in December 1999 from pigs on farm B, the source of pigs for farm A. Disc susceptibility testing showed that 57% (35/61) of the strains were resistant to ciprofloxacin and nalidixic acid. Resistant and sensitive strains were isolated from 15 pigs on farm A (Table 2.2). Paired resistant and sensitive strains were not isolated from individual pigs on farm B.

Table 2.2 Serotyping and disc susceptibility testing of porcine isolates of *C. coli*

Sample	Lab Number	Species	Serotype	Antibiotic Resistance (S or R)[1]	
				Nalidixic Acid	Ciprofloxacin
Farm A:					
2R	1754	*C. coli*	0 :5	R	R
4R	1755	*C. coli*	0 :30	R	R
6R	1756	*C. coli*	0 :59	R	R
7S	1757	*C. coli*	0 :59	S	S
8S	1758	*C. coli*	NT[2]	S	S
10R	1760	*C. coli*	0 :5	R	R
11R	1761	*C. coli*	0 :48	R	R
14R	1763	*C. coli*	0 :5	R	R
22S	1766	*C. coli*	0 :59	S	S
23R	1767	*C. coli*	0 :24	R	R
26R	1769	*C. coli*	NT	R	R
39S	1771	*C. coli*	0 :34	S	S
40R	1772	*C. coli*	NT	R	R
42S	1773	*C. coli*	0 :30	S	S
43R	1774	*C. coli*	0 :24	R	R
47S	1776	*C. coli*	NT	S	S
49S	1777	*C. coli*	NT	S	S
54R	1779	*C. coli*	0 :24	R	R
55R	1780	*C. coli*	NT	R	R
59R	1783	*C. coli*	0 :24	R	R
65S	1786	*C. coli*	NT	S	S
66R	1787	*C. coli*	0 :5	R	R
67R	1788	*C. coli*	0 :5	R	R
69S	1790	*C. coli*	NT	S	S

71R	1792	*C. coli*	0 :5	R	R
12S	1762	*C. coli*	NT	S	S
12R	1762	*C. coli*	NT	R	R
19S	1764	*C. coli*	NT	S	S
19R	1764	*C. coli*	0 :34	R	R
20S	1765	*C. coli*	0 :48	S	S
20R	1765	*C. coli*	0 :48	R	R
24S	1768	*C. coli*	0 :54	S	S
24R	1768	*C. coli*	0 :24	R	R
38S	1770	*C. coli*	NT	S	S
38R	1770	*C. coli*	0 :24	R	R
46S	1775	*C. coli*	0 :34	S	S
46R	1775	*C. coli*	0 :24	R	R
53S	1778	*C. coli*	NT	S	S
53R	1778	*C. coli*	0 :24	R	R
57S	1781	*C. coli*	NT	S	S
57R	1781	*C. coli*	0 :24	R	R
58S	1782	*C. coli*	NT	S	S
58R	1782	*C. coli*	0 :5 ; 0 :24	R	R
60S	1784	*C. coli*	0 :30	S	S
60R	1784	*C. coli*	NT	R	R
62S	1785	*C. coli*	NT	S	S
62R	1785	*C. coli*	0 :24	R	R
68S	1789	*C. coli*	NT	S	S
68R	1789	*C. coli*	0 :5 ; 0 :30	R	R
70S	1791	*C. coli*	NT	S	S
70R	1791	*C. coli*	0 :5	R	R
72S	1793	*C. coli*	0 :34	S	S
72R	1793	*C. coli*	0 :24	R	R
73S	1794	*C. coli*	NT	S	S
73R	1794	*C. coli*	NT	R	R
Farm B:					
2MF2034	2034	*C. coli*	0 :59	R	R
3MF2035	2035	*C. coli*	0 :39 ; 0 :47	R	R
5MF2036	2036	*C. coli*	0 :54	S	S
7MF2037	2037	*C. coli*	NT	S	S
9MF2038	2038	*C. coli*	0 :48	R	R
10MF2039	2039	*C. coli*	NT	R	R

[1] Antibiotic Resistance: disc susceptibility testing, sensitive (S) or resistant (R)
[2] Non-Typeable (NT)
The paired sensitive and resistant isolates from the same pigs are shown between the two, thick black lines.

A number of serotypes, including mixed serotypes, were identified in the 61 strains. The predominant serotypes were 0:24 (11/61) and 0:5 (7/61). A feature of isolates identified in this study as serotypes 24 and 5 is their uniform resistance to ciprofloxacin and nalidixic acid. Serotypes (O:48, O:54 and O:59) identified in strains from farm B were also identified in strains from farm A. A high number of the strains were non-typeable (23/61). With respect to resistant and sensitive strains isolated from the same pig: only one pair (20R and 20S) had the same serotype (O:48). A summary of serotypes and the number of strains with each type are shown in Table 2.3.

Table 2.3 Samples within each serotype.

Serotype	Sample
NT[1]	8S
	12R
	12S
	19S
	26R
	38S
	40R
	47S
	49S
	53S
	55R
	57S
	58S
	60R
	62S
	65S
	68S
	69S
	70S
	73R
	73S
	7MF2037
	10MF2039
24	23R
	24R
	38R
	43R
	46R
	53R
	54R
	57R
	59R
	62R
	72R

Table 2.3 continued.

Serotype	Sample
5	2R 10R 14R 66R 67R 70R 71R
34	19R 39S 46S 72S
48	11R 20S 20R 9MF2038
59	6R 7S 22S 2MF2034
30	4R 42S 60S
	24S 5MF2036
5; 24	58R
5; 30	68R
39; 47	3MF2035

[1] Non-typeable

2.3.2 Suitability of DNA Template for PCR

Prior to carrying out PCR experiments for *flaA* typing and other PCR based methods [Chapter 3], the suitability of extracted genomic DNA for PCR application was investigated using 16S rDNA PCR. Using primers specifically for the coding region of the 16S rRNA gene of *Campylobacter*, *Arcobacter* and *Helicobacter* (Marshall *et al.*, 1999), a 1,004 bp fragment was amplified from all of the samples, indicating the suitability of the template for PCR application. Figure 2.3 shows PCR products obtained from some of the strains.

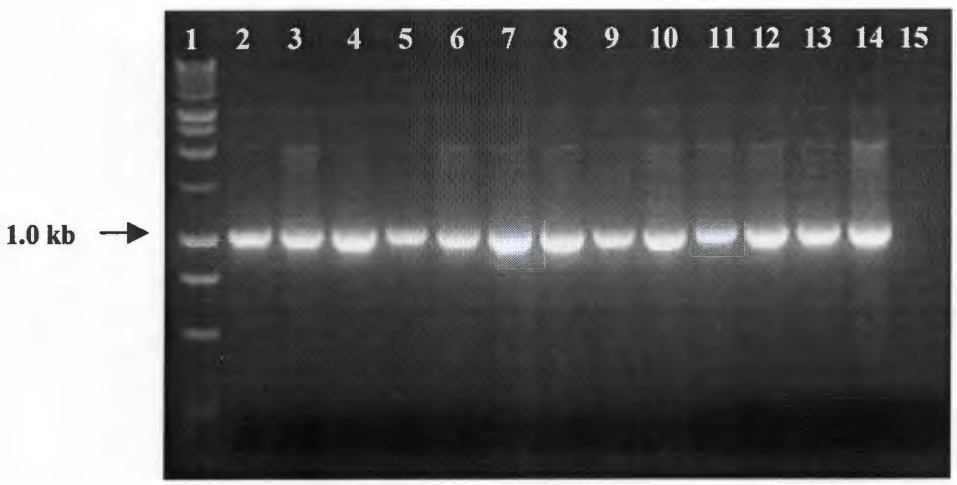


Figure 2.3 Amplification of 16S rRNA gene using *Campylobacter*, *Arcobacter* and *Helicobacter* specific primers. Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, positive control *C. jejuni*; lane 3, *C. coli* NCTC 11283; lane 4, 53S; lane 5, 62R; lane 6, 62S; lane 7, 68S; lane 8, 68R; lane 9, 70S; lane 10, 70R; lane 11, 72R; lane 12, 72S; lane 13, 73R; lane 14, 73S; lane 15, no DNA.

2.3.3 RFLP Analysis of *flaA*

Primers (Cj 431 and Cj 433) described by Ayling *et al.*, (1996) were initially used for amplification of the *flaA* gene [Table 2.1; Fig 2.2]. A product of the correct size was obtained with *C. jejuni* DNA but no product was obtained with DNA from 20/22 *C. coli* isolates. Varying the PCR conditions did not result in an amplification product. This was an unexpected result, as Ayling *et al.*, (1996) indicated that their primers could be applied to *C. coli* isolates. It should be noted that although a product of the correct size was obtained with *C. coli* NCTC11283, using these primers (Fig 2.5), amplicons were not obtained with the majority of *C. coli* isolates. To investigate further, the DNA sequences of *flaA* from *C. jejuni* (Accession number X57173) and *C. coli* (Accession number M64671) were aligned. A number of mismatches between primers and the target sequence were observed, particularly between Cj 433 (Fig 2.4). A set of primers (Cc 431 and Cc 433) which bind within the same regions as primer Cj 431 and primer Cj 433 (Fig 2.4) was designed specifically for *C. coli flaA* sequences. No PCR product was obtained using primers Cc 431 and Cc 433. As heterogeneity of the 3'end of *flaA* has been described (Harrington *et al.*, 1997), it is possible that there are mismatches between Cc 433 and the target sequence. The primer, A2, described by Nachamkin *et al.*, (1993) anneals to sequences located approximately 200 bp downstream of the binding site of primer Cc 433 [Fig 2.2]. This sequence is in a more conserved region of *flaA* and is present in *C. coli* and *C. jejuni*. Thus, primer A2 was used in conjunction with Cc 431 to amplify *flaA* of *C. coli*. Using these primers, approximately 1,700 bp amplicons were anticipated. Figure 2.5 shows the amplification products obtained using the different combinations of primers.

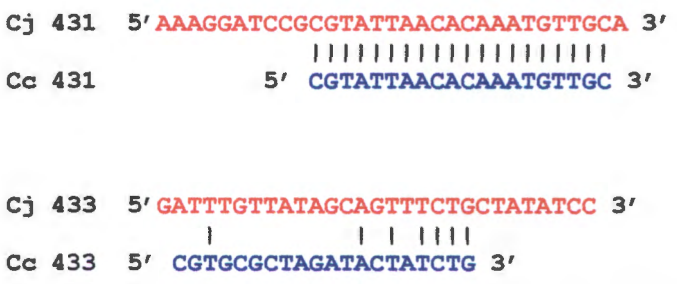


Figure 2.4 Primers specific for *C. coli flaA*. Primers Cj 431 and Cj 433 (Ayling *et al.*, 1996) were designed for the *flaA* of *C. jejuni* (red). Primers Cc 431 and Cc 433 (this study) are specific for *flaA* of *C. coli* (blue).

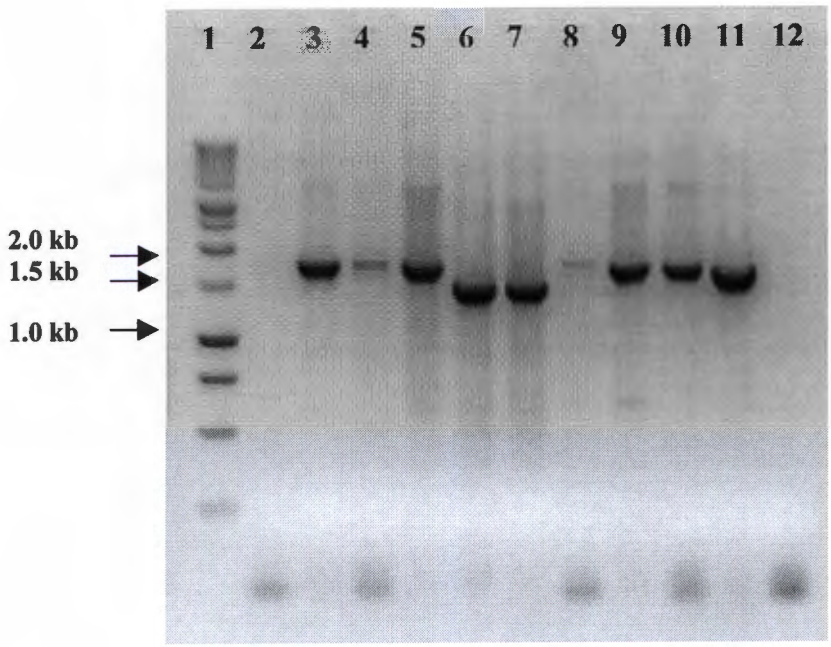


Figure 2.5 Amplicons obtained using different sets of primers.

Lane 1 – Molecular weight marker 1 kb DNA ladder (Promega).
Lane 2 – *C. jejuni*; primers Cj 431 & A2; expected product size approximately 1.7 kb.
Lane 3 – *C. jejuni*; primers Cc 431 & A2; expected product size approximately 1.7 kb.
Lane 4 – C. *coli* NCTC11283; Cj 431 & A2; expected product size approximately 1.7 kb.
Lane 5 – C. *coli* NCTC11283; Cc 431 & A2; expected product size approximately 1.7 kb.
Lane 6 – C. *coli* NCTC11283; Cj 431 & Cj 433; expected product size approximately 1.5 kb.
Lane 7 – C. *coli* NCTC11283; Cc 431 & Cj 433; expected product size approximately 1.5 kb.
Lane 8 – *C. coli* sample 62S; Cj 431 & A2; expected product size approximately 1.7 kb.
Lane 9 – *C. coli* sample 62S; Cc 431 & A2; expected product size approximately 1.7 kb.
Lane 10 – *C. coli* sample 6R; Cj 431 & A2; expected product size approximately 1.7 kb.
Lane 11 – *C. coli* sample 6R; Cc 431 & A2; expected product size approximately 1.7 kb.
Lane 12 – No DNA.

Using primers Cc 431 and A2, amplicons were obtained from 92% (56/61) of the *C. coli* isolates. Representative amplicons of the expected size (1.7 kb) are shown in Figure 2.6.

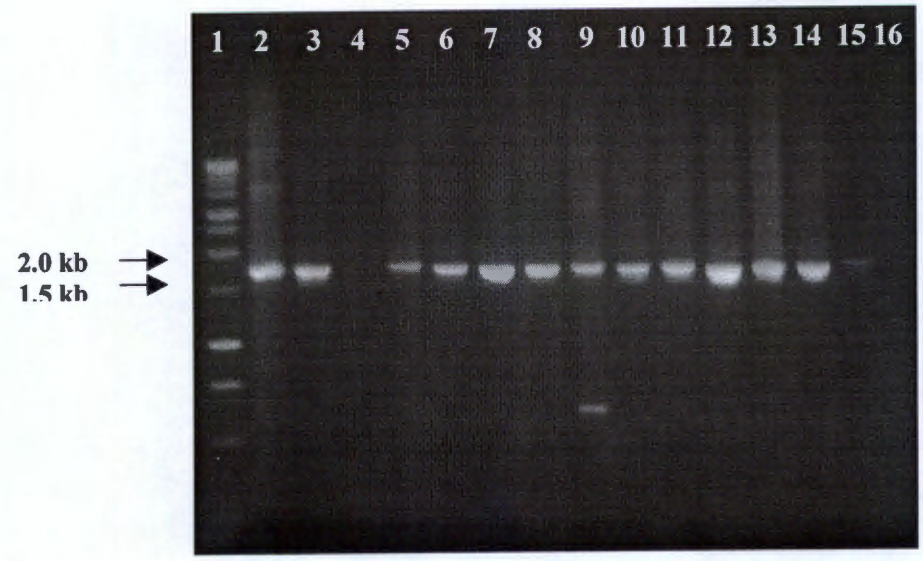


Figure 2.6 Amplification products obtained with *C. coli* DNA. Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 57R; lane 4, 58R; lane 5, 58S; lane 6, 59R; lane 7, 60S; lane 8, 62R; lane 9, 62S; lane 10, 65S; lane 11, 66R; lane 12, 67R; lane 13, 68R; lane 14, 68S; lane 15, 60R; lane 16, no DNA.

Following restriction of the *flaA* amplicons with *Dde*I, 14 profiles were obtained. A schematic diagram representing all the profiles is shown in Figure 2.7.

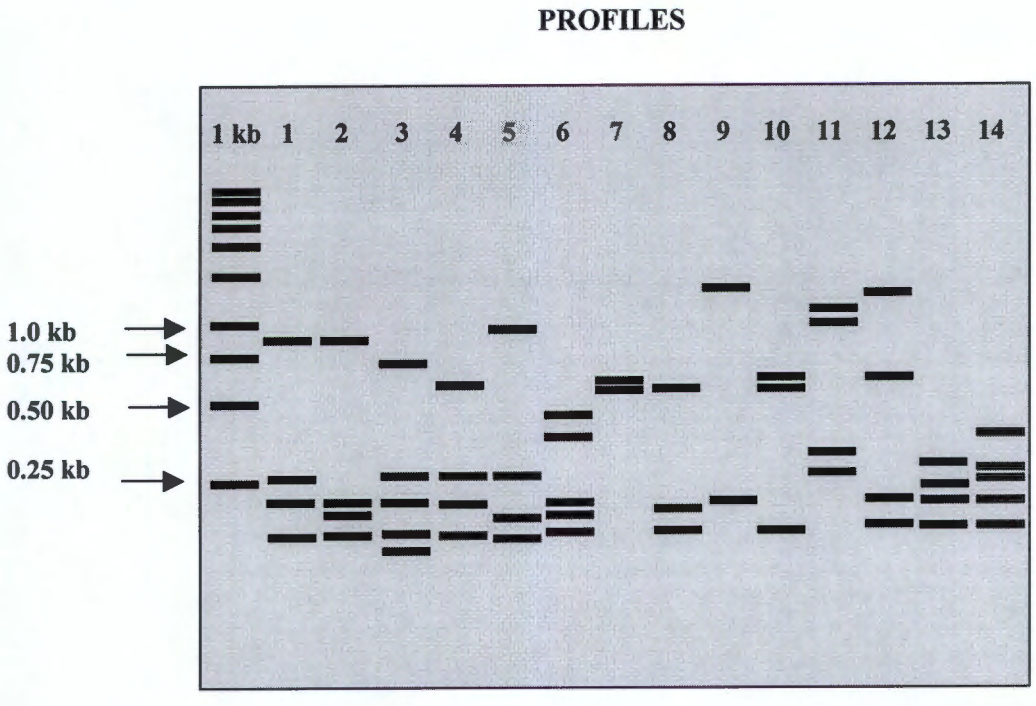


Figure 2.7 A schematic diagram representing all 14 profiles from *C. coli* isolates.

2.3.3.1 *C. coli* from Pigs on Farm A

An analysis of the profiles identified 13 distinct profiles in the 55 strains from the pigs on farm A (Figs 2.8A, B, C & D; Table 2.4). All except 2 profiles (profiles 2 and 6), were observed in both resistant and sensitive strains. Profile 1 (Fig 2.8A lanes 1, 11, 17) was the commonest profile observed (15 strains). This profile was also exhibited by *C. coli* NCTC 11283. It is interesting to note that *C. coli* NCTC 11283 was isolated from pig faeces (On, 2001). A number of serotypes were identified within profile 1 although 7 of the isolates with this profile were non-typeable (Table 2.4). The second commonest profile, profile 8 (Fig 2.8A lanes 10, 14), consists of 9 samples, of which 8 were non-typeable. Profile 6 (Fig 2.8A lanes 8, 18) consists of seven samples and is the third largest group. Two points are noteworthy with respect to isolates with this profile: They are resistant to ciprofloxacin and nalidixic acid, and they have identical serotypes (O:24). The other group (3 isolates) consisting of resistant strains only contains profile 2. These strains did not share the same serotypes (Table 2.4). The other interesting group of strains, from a typing perspective, have profile 5: 3/3 strains are serotype 48 (Table 2.4). In addition, all of the O:48 strains were resistant to ciprofloxacin and nalidixic acid.

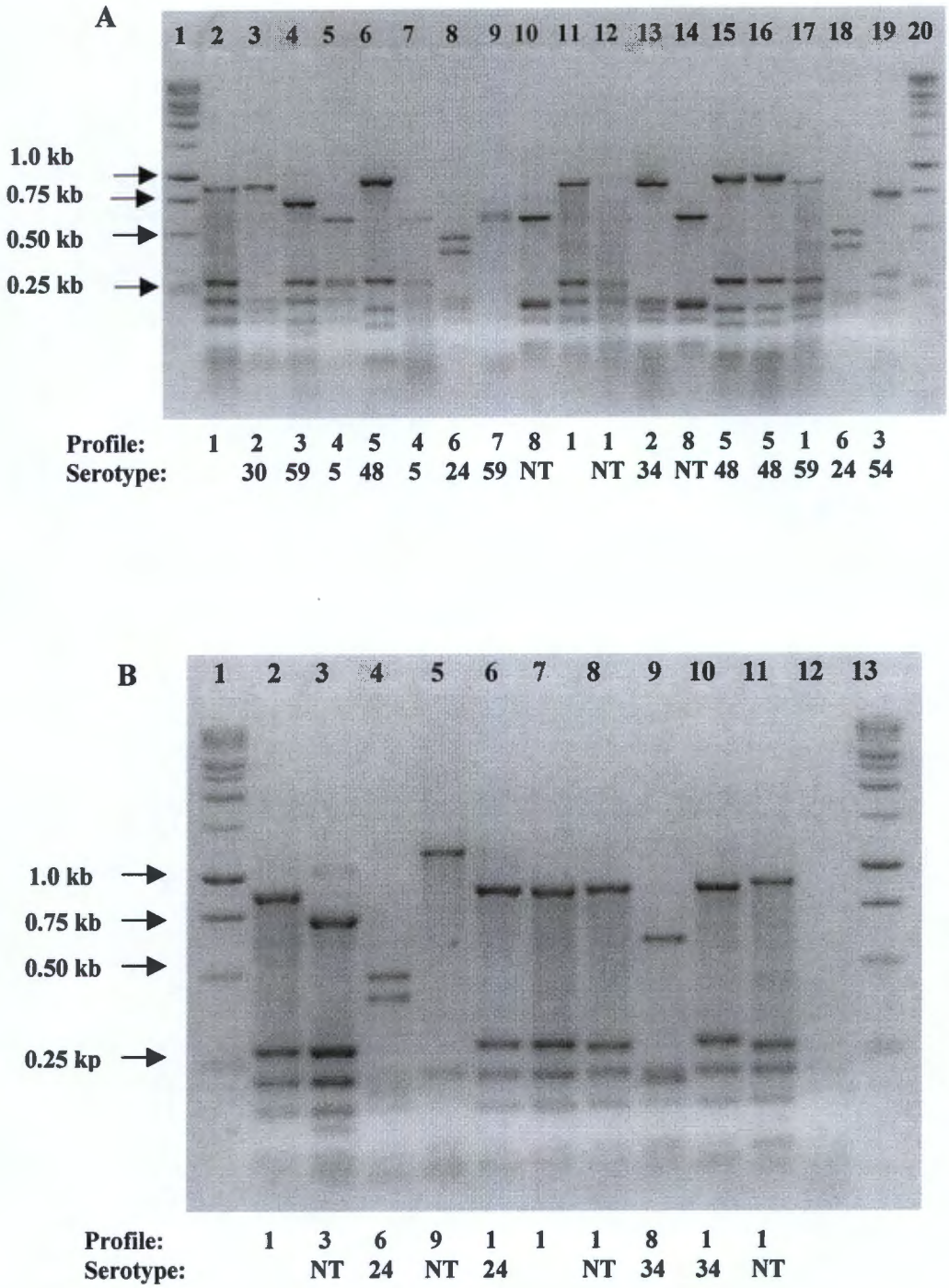


Figure 2.8 ***flaA*** **profiles among** ***C. coli*** **samples, from farm A, digested with the restriction enzyme** ***Dde*****I.** (A) Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 4R; lane 4, 6R; lane 5, 10R; lane 6, 11R; lane 7, 14R; lane 8, 23R; lane 9, 7S; lane 10, 8S; lane 11, NCTC 11283; lane 12, 12S; lane 13, 19R; lane 14, 19S; lane 15, 20R; lane 16, 20S; lane 17, 22S; lane 18, 24R; lane 19, 24S; lane 20, Molecular weight marker 1 kb DNA ladder.
(B) Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 26R; lane 4, 38R; lane 5, 40R; lane 6, 43R; lane 7, NCTC 11283; lane 8, 38S; lane 9, 39S; lane 10, 46S; lane 11, 47S; lane 12, Blank; lane 13, Molecular weight marker 1 kb DNA ladder.

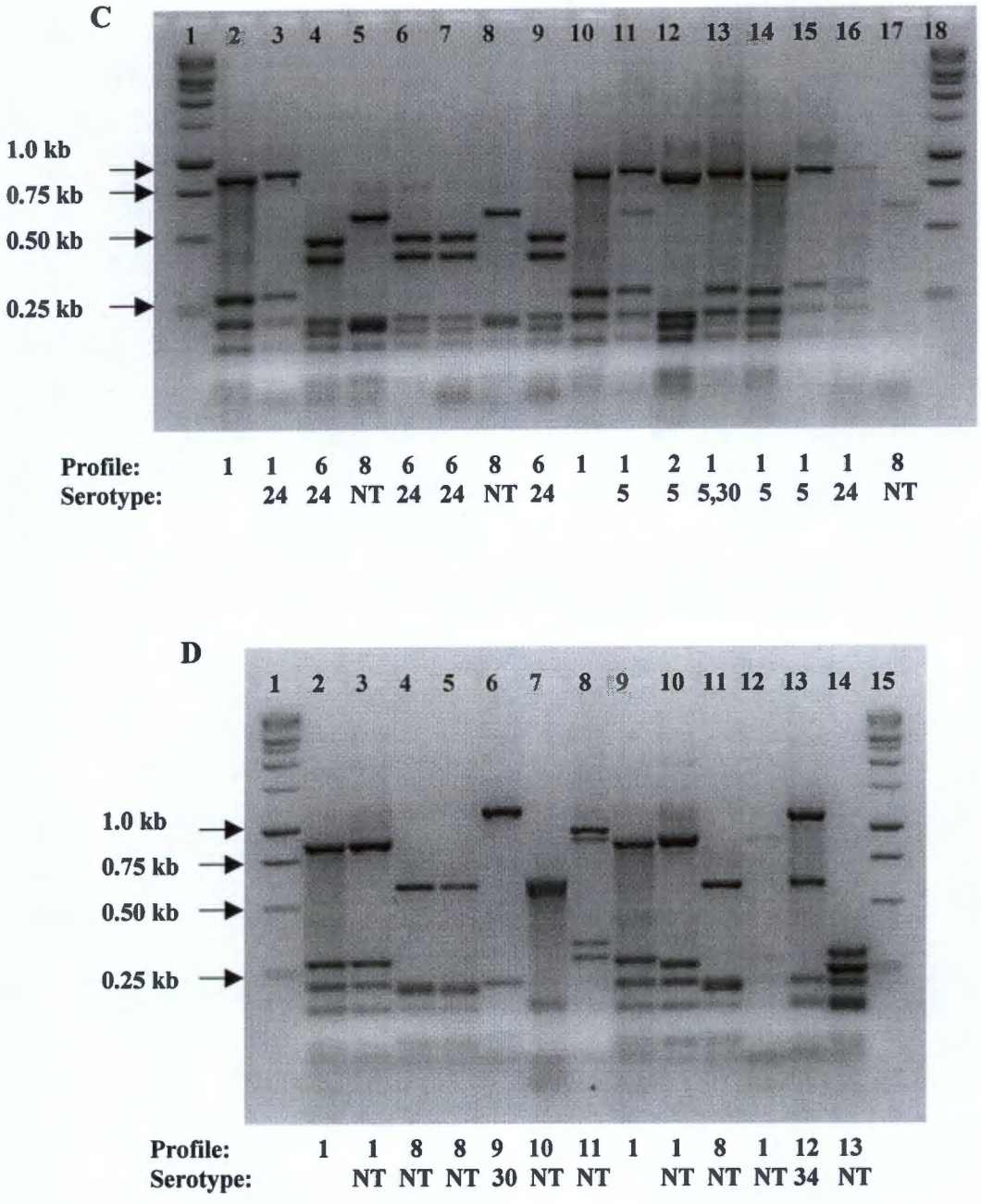


Figure 2.8 ***flaA*** **profiles among** ***C. coli*** **samples, from farm A, digested with the restriction enzyme** ***Dde*****I.** (C) Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 53R; lane 4, 54R; lane 5, 55R; lane 6, 57R; lane 7, 59R; lane 8, 60R; lane 9, 62R; lane 10, NCTC 11283; lane 11, 66R; lane 12, 67R; lane 13, 68R; lane 14, 70R; lane 15, 71R; lane 16, 72R; lane 17, 73R; lane 18, Molecular weight marker 1 kb DNA ladder.
(D) Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 53S; lane 4, 57S; lane 5, 58S; lane 6, 60S; lane 7, 62S; lane 8, 65S; lane 9, NCTC 11283; lane 10, 68S; lane 11, 69S; lane 12, 70S; lane 13, 72S; lane 14, 73S; lane 15, Molecular weight marker 1 kb DNA ladder.

Table 2.4 *flaA* profiles and Penner serotypes of *C. coli* isolates from farms A and B.

flaA Profile	Number of Samples per Profile	Serotype	Number of Resistant (R) and Sensitive (S) Isolates
1	17	NT[1] (×7) NT 59 24 (×3) 34 5,30 5 (×2) 54	R = 7 S = 8 S = 2
2	3	30 34 5	R = 3 S = 0
3	3	59 54 NT	R = 2 S = 1
4	3	5 (×2) 54	R = 2 S = 1
5	5	48 (×3) 48 (×1) NT	R = 2 R = 2 S = 1
6	7	24 (×7)	R = 7 S = 0
7	2	59 39, 47	S = 1 R = 1
8	9	NT (×8) 34	R = 3 S = 6
9	2	NT 30	R = 1 S = 1
10	1	NT	S
11	1	NT	S
12	1	34	S
13	1	NT	S
14	1	59	R

[1] Non-typeable
Blue, indicates strains are from farm A; Red, indicates strains are from farm B.

A more detailed analysis with respect to the paired resistant and sensitive isolates from 15 pigs on farm A shows that amplicons were not obtained from one of each of three pairs of isolates (12R, 46R and 58R). The profiles of the remaining 12 pairs were different from each other with the exception of 4 pairs highlighted in Table 2.5. Both 20R and 20S have *flaA* profile 5 (Fig 2.8A lanes 15 & 16) while paired samples 53R, 53S (Fig 2.8C lane 3; Fig 2.8D lane 3); 68R, 68S (Fig 2.8C lane 13; Fig 2.8D lane 10); and 70R, 70S (Fig 2.8C lane 14; Fig 2.8D lane 12) have *flaA* profile 1.

Table 2.5 Serotypes and *flaA* profiles of paired resistant and sensitive isolates.

Sample	Serotype	*flaA* Profile
12R	NT[1]	N/A[2]
12S	NT	1
19R	0 :34	2
19S	NT	8
20S	0 :48	5
20R	0 :48	5
24R	0 :24	6
24S	0 :54	3
38R	0 :24	6
38S	NT	1
46S	0 :34	1
46R	0 :24	N/A
53S	NT	1
53R	0 :24	1
57S	NT	8
57R	0 :24	6
58R	0 :5 ; 0 :24	N/A
58S	NT	8
60R	NT	8
60S	0 :30	9
62R	0 :24	6
62S	NT	10
68S	NT	1
68R	0 :5 ; 0 :30	1
70S	NT	1
70R	0 :5	1
72R	0 :24	1
72S	0 :34	12
73R	NT	8
73S	NT	13

[1] NT Non-typeable
[2] N/A No amplicon obtained.

2.3.3.2 *C. coli* from Pigs on Farm B

Profiles observed in strains from pigs on farm A were identified in strains from pigs on farm B (Table 2.4; Fig 2.9). Both of the strains with profile 1 were sensitive, the remaining strains from farm B were all resistant. Profile 14 (1 strain) is the only profile not exhibited in strains from farm A. Two of the remaining strains exhibited profile 5. As with 2/3 profile 5 strains from farm A, one of the farm B strains was O:48, and resistant to ciprofloxacin and nalidixic acid. The other could not be serotyped. The profile (profile 7) of the third isolate was also identified in one farm A strain, however their serotypes were different. In addition, the farm A isolate was sensitive whereas the farm B isolate was resistant to these antibiotics.

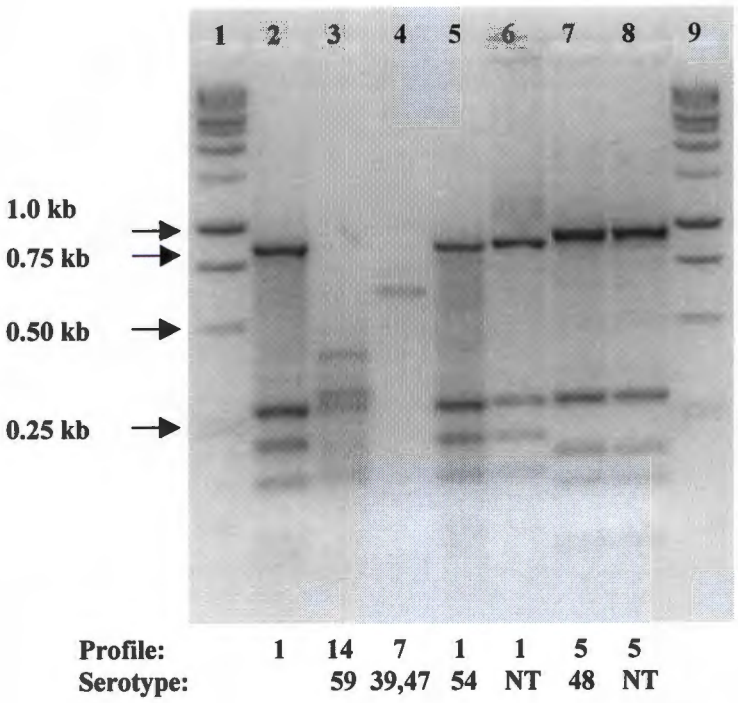


Figure 2.9 *flaA* profiles among *C. coli* samples, from farm B, digested with the restriction enzyme *Dde*I. Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, 2MF2034; lane 4, 3MF2035; lane 5, 5MF2036; lane 6, 7MF2037; lane 7, 9MF2038; lane 8, 10MF2039; lane 9, Molecular weight marker 1 kb DNA ladder (Promega).

2.3.3.3 Absence of Amplicons: An Investigation

No amplicons were obtained from 5 of the 61 isolates, all from farm A, despite modifications to the assay (annealing temperature, primer concentration, $MgCl_2$ and DNA concentration). To investigate further, PCR assays were carried out using each of the primers in separate assays. When forward primer, Cc 431, was used a product from all except one of the isolates was obtained (Fig 2.10). On the other hand, when primer A2, which anneals to the 3'end of the *flaA* gene was used, no products were obtained, suggesting a lack of complementarity between the primers and the target sequence. This was surprising since A2 anneals to the more conserved region of *flaA*.

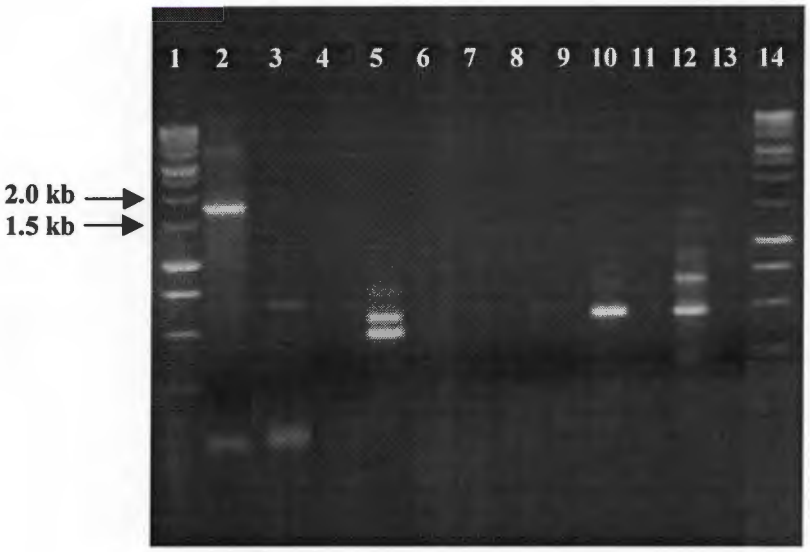


Figure 2.10 PCR assay, using single primers.

Lane 1 – Molecular weight marker 1 kb DNA ladder (Promega);
Lane 2 – *C. coli* NCTC 11283, primers Cc 431 & A2;
Lane 3 – *C. coli* sample 46R, primer Cc 431;
Lane 4 – *C. coli* sample 46R, primer A2;
Lane 5 – *C. coli* sample 12R, primer Cc 431;
Lane 6 – *C. coli* sample 12R, primer A2;
Lane 7 – *C. coli* sample 2R, primer Cc 431;
Lane 8 – *C. coli* sample 2R, primer A2;
Lane 9 – C. coli sample 58R, primer A2;
Lane 10 – *C. coli* sample 58R, primer Cc 431;
Lane 11 – *C. coli* sample 42S, primer A2
Lane 12 – *C. coli* sample 42S, primer Cc 431
Lane 13 – no DNA.
Lane 14 – Molecular weight marker 1 kb DNA ladder (Promega).

2.4 DISCUSSION

There have been many typing schemes, both phenotypic and genotypic, used in the study of *Campylobacter* spp. Phenotypic methods include techniques such as serotyping (Penner & Hennessy, 1980; Lior *et al.*, 1982;) which are useful for epidemiological purposes, however may lack the necessary discriminatory power between strains. Genotypic methods, including PFGE (Yan *et al.*, 1991; Salama *et al.*, 1992; Fujita *et al.*, 1995; Bourke *et al.*, 1996), RAPD (Williams *et al.*, 1990; Madden *et al.*, 1996) and flagellin gene typing (Nachamkin *et al.* 1993), have a higher typeability between strains but in some instances these methods maybe too complex to perform. Combinations of serotyping and genotyping methods result in a higher discriminatory power between strains, and therefore a more complete strain identification (Wassenaar & Newell, 2000). In this study, a combination of serotyping, and *flaA* typing (Nachamkin *et al.*, 1993; Ayling *et al.*, 1996) were used to type porcine isolates of *C. coli.*

Serotyping, based on the Penner & Hennessy scheme (1980), was carried out on the 61 isolates of *C. coli* (Table 2.3). Thirty-eight (62%) of the isolates were typeable while 23 (38%) were non-typeable. The number of non-typeable isolates is high; using the same serotyping scheme, Jacobs-Reitsma *et al.* (1995) reported that 19% of *Campylobacter* spp. isolates from a Dutch poultry farm were non-typeable. Similarly, Frost *et al.* (1998), reported 12% of *C. coli* isolates from clinical cases in the UK were non-typeable. Using LEP serotyping, McKay *et al.* (2001) reported that 37% of *Campylobacter* spp. were non-typeable.

A scatter of serotypes and a number of *flaA* profiles were identified in the strains typed. Nielsen *et al.*, (1997), showed that O:30 and O:46 were the commonest serotypes among porcine *C. coli.* In this study, O:24 was the most common serotype (11/38 strains) (Table 2.3). Seven of these strains had the same *flaA* profile (profile 6) (Table 2.4), indicating good correlation between this serotype and *flaA* typing, in this instance. Three of the O:24 strains had *flaA* profile 1. The remaining strains could not be typed. A feature of all serotype 24 strains is their uniform resistance to ciprofloxacin and nalidixic acid. The only other serotype consistently associated with resistance to ciprofloxacin and nalidixic acid was O:5, and as with O:24 strains, two profiles (profiles 1 and 4) were observed in the strains. Whether serotypes 5 and

24 are predisposed to developing resistance is not known. Interestingly, one isolate (58R) exhibited both these serotypes (5; 24); however, its *flaA* profile could not be determined. The possibility of antigenic shift, associated with changes in antigenic specificity related to the O side chains of the LPS molecule has been described (Mills *et al.*, 1991). They showed that *C. coli* is capable of undergoing genomic re-arrangements that lead to changes in LPS specificity and structure. The observation that most of the *flaA* profiles contain a variety of serotypes was not surprising; similar results were reported from other studies (Alm *et al.*, 1993; Burnens *et al.*, 1995; Santesteban *et al.*, 1996), suggesting that *flaA* typing is more discriminatory.

With respect to paired resistant and sensitive isolates from pigs on farm A. Each of four (20R/20S; 53S/53R; 68S/68R; 70S/70R) of the 12 pairs had the same *flaA* profile (Table 2.5). Profile 1 was identified in 3 pairs, while profile 5 was exhibited in 1 pair (20R/20S). In addition to having the same *flaA* profile, the latter pair had identical serotypes (O:48).These data suggest that resistant mutants from a previously sensitive population have been selected in these pigs.

Resistant and sensitive strains were isolated from farm B. Both serotype (O:59, O:54, and O:48) and *flaA* profiles (profiles 1, 5, and 7) (Table 2.4) observed in resistant strains from farm A were exhibited by resistant strains from farm B, suggesting a relatedness between some strains from the two farms. As farm B is the supplier of pigs to farm A, it is may be that the pigs on farm A were received already colonised with resistant strains. On the other hand, may be resistance arose independently on the two farms.

Overall, there was variable relationship between Penner serotype and *flaA* profile. A single profile could consist of up to 6 serotypes (Table 2.4; Profile 1), however in general profiles consisted of one, two or three serotypes. Conversely, a single serotype comprised a number of *flaA* profiles. This was not surprising as other studies have shown variable correlation between serotyping and *flaA* typing (Alm *et al.*, 1993; Burnens *et al.*, 1995; Santesteban *et al.*, 1996). Although studies have demonstrated direct correlation between flagellin typing and serotyping), the correlations are generally weak (Nachamkin *et al.*, 1993; Owen *et al.*, 1993b; Burnens *et al.*, 1995; Nachamkin *et al.*, 1996).

Although significant heterogeneity among the *flaA* gene of *C. jejuni* has been shown in previous research (Thornton *et al.*, 1990; Nachamkin *et al.*, 1993), Thornton *et al.*, (1990) suggested that *Campylobacter* flagellins are more conserved than is indicated by the HL serotyping scheme of Lior *et al.*, (1982). This is contradicted by results from Nachamkin *et al.* (1993), suggesting that at the nucleotide level there is more heterogeneity than is suggested by HL serotyping. Results from this study support those of Nachamkin *et al.*, (1993) for the Penner scheme of serotyping. The variety of profiles indicates that there is greater heterogeneity within the *flaA* gene than indicated by the Penner scheme.

Using a variety of primers, amplicons were not obtained with DNA from 5 isolates. Significantly, in the cases investigated, this was due to the lack of primer binding to the 3'end of *flaA*. Heterogeneity of the 3'end of *flaA* could explain this result. There is strong evidence for intergenomic recombination between *flaA* genes of different strains (Wassenaar *et al.*, 1995; Harrington *et al.*, 1997). Furthermore, Harrington *et al.* (1997) provided evidence for intragenomic recombination between the *flaA* and *flaB* genes of individual strains. The frequency of such recombination in natural circumstances is unknown but clearly such heterogeneity would disadvantage *flaA* typing. For this reason Newell *et al.*, (2000) has suggested *flaA* typing needs to be supported by additional phenotypic or genotypic methods for accurate analysis. Interestingly, *C. coli* NCTC 11283 isolated in 1970 (On, 2001) exhibited profile 1 suggesting *flaA* stability.

CHAPTER 3

MOLECULAR BASIS OF FLUOROQUINOLONE RESISTANCE

3.1 INTRODUCTION

In this study, 57% (35/61) of the *C. coli* isolates were resistant to ciprofloxacin and nalidixic acid, while 43% (26/61) were susceptible. This chapter describes the studies carried out to determine the molecular basis of the resistance.

3.1.1 Treatment

Diarrhoea resulting from *C. jejuni* and *C. coli* is generally a self-limiting disease lasting up to 5 days. Infection can persist longer and may develop into severe forms, especially in children and immunocompromised patients (Blaser *et al.*, 1979; Blaser *et al.*, 1984). Early treatment with antibiotics is recommended to reduce severity and duration of infection. Erythromycin, fluoroquinolones and tetracyclines are the antimicrobial agents commonly used to treat severe cases of *C. jejuni* infection (Blaser, 1997; Altkreuse *et al.*, 1999). Fluoroquinolones are often the treatment of choice for adults and are also prescribed for prophylactic treatment against traveller's diarrhoea (Taylor *et al.*, 1991; Nachamkin, 1993; Piddock, 1995). Fluoroquinolone resistance has been described in *Campylobacter* spp. (Piddock, 1995).

3.1.2 Fluoroquinolone Resistance

Fluoroquinolones were introduced in the early 1980s and were hailed with great enthusiasm as a new class of antibiotic for antibiotic therapy. Fluoroquinolones are active against most major pathogens causing bacterial enteritis, and had good in vitro activity against all *Campylobacter* spp. and against members of the family *Enterobacteriaceae* (Taylor *et al.*, 1985; Wolfson & Hooper, 1989; Bryan *et al.*, 1990). Besides their potent in vitro activity against susceptible bacteria, fluoroquinolones can achieve high concentrations in the gastrointestinal tract (Du Pont *et al.*, 1987). Unfortunately, since the late 1980s, resistance to fluoroquinolones in campylobacter isolates has been increasing (Piddock, 1995), especially in Europe following the introduction of enrofloxacin (a derivative of ciprofloxacin) into veterinary medicine and, less importantly, the use of fluoroquinolones in human medicine.

Resistance in *Campylobacter* spp. can arise in vivo, sometimes even after only one or two administrations of fluoroquinolones (Adler *et al.*, 1991). The induction of fluoroquinolone resistance during antibiotic therapy is recognised and frequently reported (Adler *et al.*, 1991; Ellis *et al.*, 1995; Segreti *et al.*, 1992). Fluoroquinolone resistant *Campylobacter* continues to be a problem (Piddock, 1995), resistance has been reported in a number of countries including the UK (Gaunt & Piddock, (1996), the USA (Smith *et al.*, 1999; Zirnstein *et al.*, 1999), the Netherlands (Koenraad *et al.*, 1995), Spain (Sáenz *et al.*, 2000), Sweden (Sjögren *et al.*, 1997), and Thailand (Kuschner *et al.*, 1995).

3.1.3 Mode of Action of Fluoroquinolones

Fluoroquinolones, including ciprofloxacin, ofloxacin, and norfloxacin are a subgroup of quinolones derived from naladixic acid and possess a fluorine at the C-6 position of the quinolone structure. These microbial agents target the type II topoisomerases DNA gyrase and topoisomerase IV (Drlica & Zhao, 1997). The enzymes are essential for DNA replication, recombination and transcription. Both topoisomerases are tetramers composed of two subunits arranged into A_2B_2 holoenzymes: gyrase is $GyrA_2GyrB_2$, and topoisomerase IV is $ParC_2ParE_2$ (Drlica & Zhao, 1997). DNA gyrase catalyses the negative supercoiling of relaxed or positively supercoiled, double stranded, covalently closed circular DNA (Gellert *et al.*, 1977; Maxwell, 1992) while topoisomerase IV is involved in separation. Fluoroquinolones form a ternary complex with gyrase and the DNA rather than directly binding to the gyrase or DNA (Willmott & Maxwell, 1993). The fluoroquinolone-gyrase DNA complex prevents DNA replication and transcription resulting in termination of cell growth. Cleavage of double stranded DNA follows complex formation resulting in cell death (Drlica & Zhao, 1997; Kampranis & Maxwell, 1998).

3.1.4 Mechanisms of Resistance to Fluoroquinolones

Mutations in the genes *gyrA* and/or *parC* encoding the A subunits of the enzymes are the commonest mechanisms observed in clinical isolates of gram-negative bacteria (Everett & Piddock, 1997). In *E. coli*, most mutations resulting in fluoroquinolone resistance have been shown to map to a small region termed the quinolone resistance determining region (QRDR) near the N terminus of the DNA gyrase GyrA subunit between codons 67 and 106 (Yoshida *et al.*, 1990). Similarly, Wang *et al.* (1993)

demonstrated that mutations in the corresponding region of *gyrA* from *C. jejuni*, specifically at Thr-86, were associated with fluoroquinolone resistance (16-64 μg/ml).

In the same study, different mutations (Asp-90 and Ala-70) were also identified but these resulted in only modest increase in ciprofloxacin MIC (1 and 4 μg/ml) (Wang *et al.*, 1993). Other studies have identified the same mutation (Thr-86) in humans and chicken isolates of *C. jejuni* (Everett *et al.*, 1995; Ruiz *et al.*, 1998) and in clinical isolates with ciprofloxacin MICs of 34-125 μg/ml (Gibreel *et al.*, 1998). In the same study a comparison of the QRDR of *parC* from ciprofloxacin resistant *C. jejuni* and susceptible *E. coli* identified a mutation leading to the incorporation of arginine instead of glutamine at position 139 in ParC of *C. jejuni*. It was suggested that the combination of mutations (*gyrA*/*parC*) played a role in the resistance. However, this interpretation may be flawed because the same mutation was identified in susceptible *C. jejuni* isolates (Gibreel *et al.*, 1998).

3.2 EXPERIMENTAL PROTOCOL

3.2.1 Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) of ciprofloxacin were carried out on all the isolates in this study [Table 2.2]. The MICs of ciprofloxacin were determined using E-strips (AB Biodisk, Solna, Sweden) by a medical technologist in the clinical microbiology laboratory, Groote Schuur Hospital. The E-test constitutes a predefined exponential gradient of antibiotic concentrations immobilised along a rectangular plastic test strip. The strip is applied to the surface of an agar plate inoculated with the isolate to be tested. Following incubation overnight, a drop shaped zone of inhibition is seen. The edge of the zone intersects the graded test strip at the inhibitory concentration of the antibiotic. The MIC is read where the inhibition ellipse intersects the scale on the strip. The MIC is read as the lowest concentration of antibiotic required to cause a marked decrease in growth.

3.2.2 Amplification of the *gyrA* Gene

PCR [section 2.2.6 and Fig 2.1] was utilised for the amplification of the QRDR of the *gyrA* gene. Chromosomal DNA was purified as stipulated [section 2.2.2] and diluted to approximately 200 ng/µl for the assays.

3.2.2.1 Primers

Primers used for the amplification are shown in Table 3.1. Primer sets were synthesised by GIBCO BRL, Custom Primer services (Life Technologies). Initially, the degenerate primer set, GyrA1 and GyrA2, based on the nucleotide sequence of *C. jejuni* (Wang *et al.*, 1993), was used for the amplification of a 449 bp DNA fragment encoding the amino-terminal of GyrA. During the course of these studies, primers specific for amplification of the QRDR of *C. coli* (505 bp product) were published (Zirnstein *et al.*, 2000) and these were used in later experiments. Figure 3.1 is a schematic diagram of the *gyrA* gene showing the annealing sites of the primer sets.

Table 3.1 Primers used in the amplification of QRDR of the *gyrA* gene.

Primer Name	Sequence (5' to 3')[1]	Direction	Reference
GyrA1	GTI AGI GAT GGI TTI AAG CCI GTI CAT	Forward	Wang *et al.*, (1993)
GyrA2	GTG IGG IGG TAT GTT IGT IGC CAT	Reverse	Wang *et al.*, (1993)
GZgyrACcoli3F	TAT GAG CGT TAT TAT CGG TC	Forward	Zirnstein *et al.*, (2000)
GZgyrACcoli4R	GTC CAT CTA CAA GCT CGT TA	Reverse	Zirnstein *et al.*, (2000)

[1] Where is I is deoxyinosine

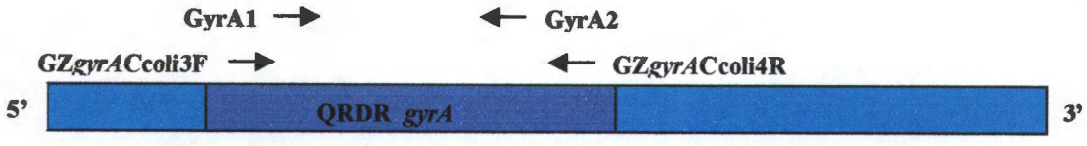


Figure 3.1 A schematic representation of the PCR primer binding sites.
GyrA1, GyrA2; and GZ*gyrA*Ccoli3F, GZ*gyrA*Ccoli4R; were designed to amplify the QRDRs of *C. jejuni* and *C. coli* respectively (Wang *et al.*, 1993; Zirnstein *et al.*, 2000).

3.2.2.2 PCR Assay Conditions

Amplification using GyrA1 and GyrA2 was carried out according to Wang *et al.*, (1993) using a modified protocol: Initial denaturation was performed at 95°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 54°C for 1 minute, 72°C for 1 minute. A final extension took place at 72°C for 7 minutes.

PCR cycling conditions using GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli4R were as follows: initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, with a final step at 72°C for 5 minutes (Zirnstein *et al.*, 1999). All PCR thermocycling reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400.

When necessary, PCR products were purified, following agarose gel electrophoresis, using the phenol-squeeze method [section 3.2.4].

3.2.3 Amplification of *parC*

3.2.3.1 Primers

Primers used for the amplification of the QRDR *parC* are shown in Table 3.2. Primer sets were synthesised by GIBCO BRL, Custom Primer services (Life Technologies). Primers are based on the QRDR *parC* of *C. jejuni* and their binding sites are shown in Figure 3.2.

Table 3.2 Primers used for *parC* amplification.

Primer Name	Orientation (Forward/Reverse)	Sequence (5'→3')	Reference
P1	Forward	TGGGATCCAAACCTGTTCAGCGCCGCATT	Gibreel *et al.*, (1998)
P2	Reverse	CGGAATTCGTGGTGCCGTTAAGCAAA	Gibreel *et al.*, (1998)
CjparCR2	Reverse	ATATCCAGCAGCTGATCG	This study
CjparCF2	Forward	GTATGCGATGTCTGAACTGG	This study
CjparCR6	Reverse	GCCACTTCACGCAGGTTATG	This study

Figure 3.2 QRDR (bases 140-919) of *C. jejuni parC* (accession number Y18300). Arrows indicate the primers used in PCR assays.

3.2.3.2 PCR Assay Conditions

Initially, amplification of the QRDR of *parC* was carried out according to the method of Gibreel *et al.*, (1998): Initial denaturation was performed at 94°C for 3 minutes followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute, with a final step at 72°C for 5 minutes. Optimisation/modification of the assay included the following: variation of annealing temperature (40°C to 60°C), $MgCl_2$ concentration and primer concentration.

3.2.4 DNA Purification

DNA purification was based on the phenol-squeeze method of Sambrook *et al.*, (1989). PCR products were electrophoresed [section 2.2.4] and the band of interest was excised from the agarose gel. The agarose slice was finely chopped up, and placed in an Eppendorf tube. An equal volume of phenol (Appendix A) was added and the mixture was placed at -70°C for 10 minutes. The solution was centrifuged at 14,000 rpm for 10 minutes followed by the removal of the supernatant. An equal volume of chloroform-isoamyl (Appendix A) was added to the supernantant to remove any residual phenol and agarose. Centrifugation was carried out and the previous step was repeated. The DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate (NaAC) pH 5.2 (Appendix A) and 3× volumes of 100% ethanol (EtOH). The DNA was pelleted by centrifugation at 14,000 rpm for 10 minute. The resulting DNA pellet was washed twice with 70% EtOH, dried and resuspended in 25µl of dH_20. DNA concentration was determined as in section 2.2.3.

3.2.5 Cloning

To facilitate characterisation and to provide glycerol stocks (30% v/v, 70% 2× YT), selected PCR products were cloned and the recombinant plasmids were introduced into *E. coli* by transformation. Circular plasmid vectors were cleaved with a restriction endonuclease leaving the ends free to ligate with the appropriate ends of DNA to be cloned (insert), thereby constructing a recombinant plasmid. Recombinant plasmids were introduced into bacterial cells, which had been made competent for the uptake of DNA. Recombinants containing the DNA of interest were characterised.

3.2.5.1 Preparation of Vector DNA (pUC19)

To obtain blunt-ends, the vector, pUC19 was digested (within the multiple cloning site (Appendix C)) with 20U of *Sma*I (CCC↓GGG) (Boehringer Mannheim; Mannheim, Germany) at 37°C for 2 hours, in the reaction conditions specified by the manufacturers. The linearised plasmid was visualised following agarose gel electrophoresis [section 2.2.4] and purified [section 3.2.4].

3.2.5.2 Preparation of Insert DNA

The PCR products were purified [section 3.2.4] and blunted using a DNA blunting kit (Amersham International, Buckinghamshire UK) to remove any dATP residues incorporated at the 3' end of the amplified product due to the template-independent terminal transferase activity of *Taq* DNA polymerase. Following the removal of the overlaps by the 3'-5' activity of the T4 DNA polymerase, supplied with the blunting kit, the DNA mixture was placed on ice in preparation for the ligation reaction.

3.2.5.3 Ligation Reaction

Ligation was carried out according to the protocol supplied with the DNA blunting kit. Ligase and reaction buffer (supplied with the kit) were added to approximately 500-800 ng of blunted insert and 100 ng of *Sma*I-digested vector. Ligation took place at 16°C for approximately 8 hours.

3.2.5.4 Preparation of Competent Cells

The $CaCl_2$ shock procedure of Dagert and Ehrlich (1979) was used to make *E. coli* LK111 cells competent for the uptake of DNA. A volume of 5 ml of 2× YT broth (Appendix A) was inoculated with *E. coli* LK111 and incubated with shaking at 37°C O/N. Following incubation, the culture was diluted 1/100 in 100ml 2× YT broth. The diluted culture was propagated to early log phase corresponding to an OD_{600} of 0.2-0.4. The cells were harvested by centrifugation (4000-5000 rpm) for 5 minutes in a Beckman J2-21 centrifuge with a JA-10 rotor at 4°C. The cells were gently resuspended in ½ culture volume of 0.1M ice-cold $CaCl_2$. The cells were kept on ice for 2 hours and then collected as described previously, and resuspended in 1/10 culture volume 0.1M $CaCl_2$, inducing the transient state of competence. Ice-cold sterile glycerol was added to a final concentration of 10% (v/v) to allow for indefinite storage of cell. Aliquots of competent cells were stored at -70°C.

3.2.5.5 Transformation

The ligation mix (5 µl) was added to competent *E. coli* LK111 (100 µl) and placed on ice for 30 minutes to facilitate DNA binding to the bacterial cells. A heat shock was applied at 42°C for 2 minutes to facilitate uptake of DNA by the cell. To encourage expression of the ampicillin resistance gene carried by pUC19 (Appendix C), 0.9 ml of 2× YT broth was added to the transformed cells followed by incubation for 1 hour

at 37°C. The expression mix (200µl) was plated onto 2× YT agar plates containing 1 ml X-gal (5-bromo-4-chloro-β-D-galactoside, 40 mg/ml, Appendix A), 0.1 ml of IPTG (Isopropyl-β-D-thio-galactopyranoside, 200 mg/ml, Appendix A), 0.2 ml of ampicillin (100 mg/ml, Appendix A) per 200 ml of 2X YT agar (Appendix A). The plates were inverted and incubated overnight at 37°C. Cells used were shown to have a transformation efficiency of 1.6×10^6.

3.2.5.6 Screening of *E. coli* LK111 Containing Recombinant Plasmids

Selection of recombinants took place on two levels. Firstly, ampicillin was used to select for cells carrying pUC19, which encodes ampicillin resistance. The second level of selection is based on the presence or absence of a functional β-galactosidase gene. The *lacZ* gene on pUC19 encodes the amino terminus of the β-galactosidase (the α fragment), while *E. coli* LK111 contains the carboxyl terminus of the β-galactosidase (the β fragment). Disruption of the *lacZ* gene as a result of ligation of insert DNA into the multiple cloning site (MCS) within the *lacZ* gene interrupts production of the α fragment. It follows that α complementation cannot take place and that active β-galactosidase is not present within the cell. The presence of β-galactosidase is detected by adding X-gal and IPTG to the nutrient agar. IPTG induces *lacZ* production, and X-gal, a substrate for β-galactosidase, is cleaved producing a blue colony. In the absence of β-galactosidase, X-gal is not cleaved and the colonies appear white, suggesting that the cells contain a recombinant plasmid. On the other hand, blue colonies indicate that the bacterial cells contain vector only (Sambrook *et al.*, 1989).

3.2.5.7 Small-Scale Plasmid Preparation

Small-scale plasmid preparations were performed using the method based on Ish-Horowicz & Burke (1981). A 5ml 2× YT broth (ampicillin selection at 100 µg/ml) was inoculated with a single possible recombinant colony. The inoculated broth was incubated at 37°C with shaking for 16 hours. The cells were harvested by centrifugation at 14,000 rpm for 15 minutes and resuspended in 0.2 ml of glucose/ Tris-Cl/EDTA solution I (Appendix A) to create spheroplasts. The cells were lysed with SDS, and the resulting DNA was denatured with NaOH (Solution II, Appendix A). Selective renaturation of plasmid DNA was facilitated by the addition of 3M potassium acetate/glacial acetic acid solution III (Appendix A) at 4°C for 5 minutes. The white flocculate containing cellular debris and chromosomal DNA was removed by centrifugation. The supernatant was recovered following centrifugation and

isopropanol (0.6 volume) was added to precipitate the DNA. The DNA was pelleted, washed in 80% ethanol and dissolved in 50 µl of dH_2O.

3.2.6 DNA Sequencing

DNA sequencing was carried out using Automated sequencing (Pharmacia Biotech AB S-751 82 Uppsala Sweden) in the Department of Molecular Biology, University of Cape Town.

3.2.6.1 Sequence Analysis

Sequence similarity searches for nucleic acids was carried out against existing nucleotide and databases using the Basic Local Alignment Search Tool, Blast (Altschul *et al.*, 1990); at the National Centre for Biotechnology Information (NCBI), National Institute of Health. DNA sequences of interest were analysed by DNAMAN version 4.0 (Lynnon Biosoft) computer analysis software for nucleic acid and amino acid sequences.

3.3 RESULTS

3.3.1 Antimicrobial Susceptibility

The MICs of ciprofloxacin were determined for all *C. coli* isolates using E-strips and are shown in Table 3.3. The lowest MIC observed is 0.016 μg/ml and the highest MIC, ≥32 μg/ml. MIC values for ciprofloxacin can be defined as: sensitive ≤ 1 μg/ml; intermediate between 1 and 4 μg/ml; and resistant ≥ 4 μg/ml (NCCLS, 1998b).

Table 3.3 Ciprofloxacin MICs of *C. coli* isolates.

Sample	Antibiotic Resistance (S or R)[1]		Ciprofloxacin MIC (μg/ml)
	Naladixic Acid	Ciprofloxacin	
Farm A:			
2R	R	R	≥32
4R	R	R	≥32
6R	R	R	≥32
7S	S	S	0.064
8S	S	S	0.25
10R	R	R	32
11R	R	R	4
14R	R	R	≥32
22S	S	S	0.125
23R	R	R	32
26R	R	R	≥32
39S	S	S	0.064
40R	R	R	≥32
42S	S	S	0.064
43R	R	R	≥32
47S	S	S	0.064
49S	S	S	0.125
54R	R	R	12
55R	R	R	16
59R	R	R	≥32
65S	S	S	0.064
66R	R	R	≥32
67R	R	R	≥32
69S	S	S	0.125
71R	R	R	≥32
12S	S	S	0.5
12R	R	R	≥32
19S	S	S	0.032
19R	R	R	≥32

20S	S	S	0.125
20R	R	R	≥32
24S	S	S	0.25
24R	R	R	≥32
38S	S	S	0.064
38R	R	R	≥32
46S	S	S	0.064
46R	R	R	≥32
53S	S	S	0.032
53R	R	R	≥32
57S	S	S	0.125
57R	R	R	24
58S	S	S	0.032
58R	R	R	≥32
60S	S	S	0.016
60R	R	R	8
62S	S	S	0.032
62R	R	R	≥32
68S	S	S	0.064
68R	R	R	≥32
70S	S	S	0.125
70R	R	R	≥32
72S	S	S	0.064
72R	R	R	≥32
73S	S	S	0.064
73R	R	R	2
Farm B:			
2MF2034	R	R	≥32
3MF2035	R	R	≥32
5MF2036	S	S	0.25
7MF2037	S	S	0.032
9MF2038	R	R	4
10MF2039	R	R	4

[1] Antibiotic Resistance: disc susceptibility testing, sensitive (S) or resistant (R)
The paired sensitive and resistant isolates from the same pigs are shown between the two, thick black lines.

3.3.2 Amplification of *gyrA* Using Primers GyrA1 and GyrA2

Mutations in *gyrA* associated with fluoroquinolone resistance in *C. jejuni* have been described (Wang *et al.*, 1993; Everett *et al.*, 1995; Ruiz *et al.*, 1998). Although fluoroquinolone resistant *C. coli* have been described, studies on the molecular basis of this resistance were published (Zirnstein *et al.*, 2000) after this study commenced. Thus, in the first instance, degenerate primers (GyrA1 and GyrA2), which have been used to amplify the QRDR *gyrA* gene of *C. jejuni* UA580 and *C. fetus* subsp. *fetus* (Wang *et al.*, 1993; Taylor & Chau, 1997), were used in PCR assays to amplify the corresponding region in *C. coli* [section 3.2.2.2]. A product of the expected size (449 bp) was obtained for *C. jejuni* but no product was obtained for 77% (10/13) of the *C. coli* samples tested. Moreover, when a product of the expected size was amplified, an additional larger product was obtained. Representative products obtained using primers GyrA1 and GyrA2 are shown in Figure 3.3. This data suggested mismatches between primer(s) and the target sequence, preventing amplification. To obtain DNA sequencing data of the QRDR of *gyrA* of *C. coli*, an amplicon of the correct size (449 bp) was excised from the gel and DNA was purified [section 3.2.4] and subsequently sequenced directly. As unsatisfactory DNA sequencing was obtained, a representative amplicon was cloned and sequenced [sections 3.2.5 and 3.2.6].

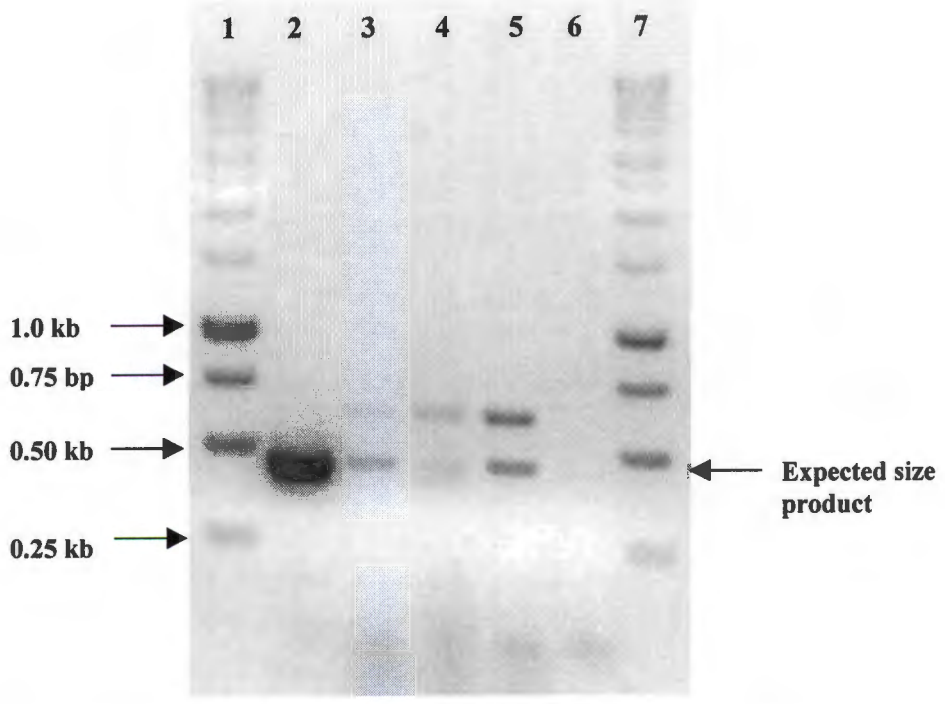


Figure 3.3 Amplification of the QRDR of *gyrA* of *C. coli* isolates using GyrA1 and GyrA2 (Chau & Taylor (1997). Lanes 1 and 7 Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. jejuni*; lane 3, 6R; lane 4, 7S; lane 5, 10MF2039; lane 6, No DNA.

3.3.3 Cloning PCR Products

Amplification products from one of the strains (10MF2039) were cloned into pUC19 [section 3.2.5]. Following transformation [section 3.2.5.5] more than 1000 white, ampicillin resistant colonies were obtained, suggesting that pUC19 had been taken up by *E. coli* and that the *lacZ* gene had been interrupted, thereby preventing α-complementation. Small-scale plasmid preparation [section 3.2.5.7] was carried out on 25 white colonies. Following digestion with *Eco*RI and *Bam*HI to release the insert, the fragments were separated by agarose gel electrophoresis [section 2.2.4]. As shown in Figure 3.4, only 2 of the putative recombinants contained a 449 bp insert. The remaining white colonies probably contain pUC19 which has a point mutation in the MCS, disrupting α-complementation. One recombinant containing an insert was selected: DNA was extracted [section 3.2.5.7] and submitted for sequencing [section 3.2.6].

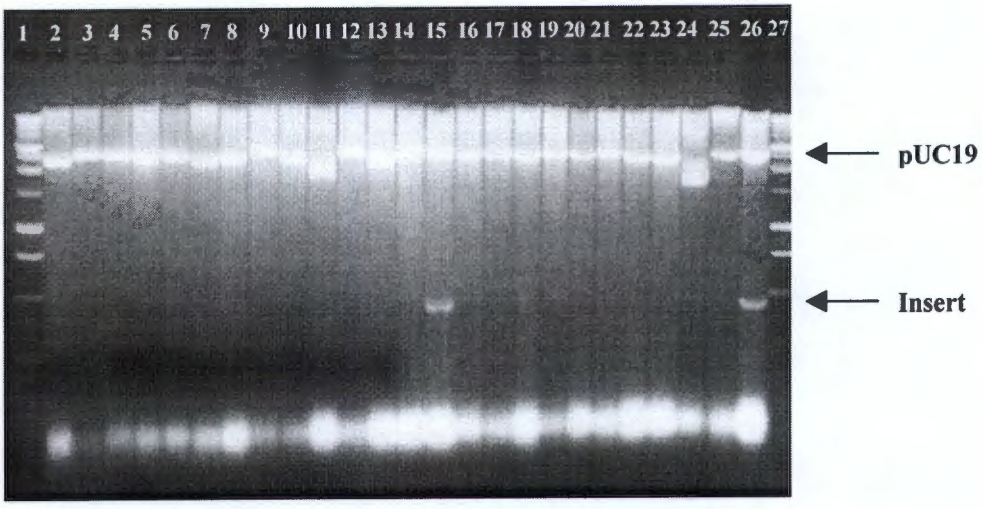


Figure 3.4 Analysis of putative recombinant plamids following restriction endonuclease digestion with *Eco*RI and *Bam*HI. Lanes 1 and 27, Molecular weight marker 1 kb DNA ladder (Promega); lanes 2-14 and 16-25, vector (2, 686 bp) DNA; lanes 15 and 26, vector DNA and 10MF2039 insert (449 bp).

3.3.3.1 Sequence Analysis of QRDR of *gyrA* of Sample 10MF2039

The nucleotide sequence of the amplified QRDR of *C. coli gyrA* MF2039 was smaller (444 bp) than that observed by Taylor & Chau, (1997) for *C. fetus* subsp. *fetus* (449 bp), using the primers GyrA1 and GyrA2. The nucleotide sequence was aligned with the corresponding region of *gyrA* from *C. jejuni* (accession number L04566). An analysis of the data shows a 77% DNA similarity (Figure 3.5).

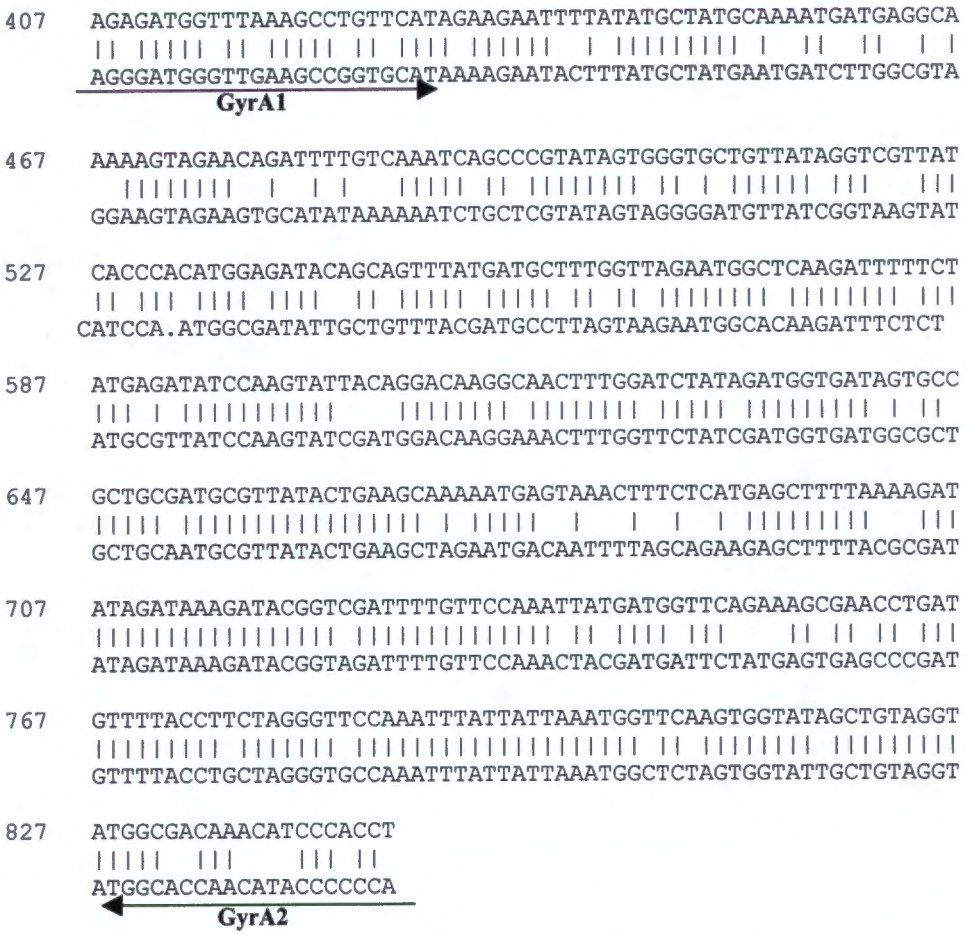


Figure 3.5 Alignment of the QRDR *gyrA* of sample 10MF2039 and the corresponding region of *gyrA* of *C. jejuni* (accession number AF092101). The top sequence is *C. jejuni* and the bottom sequence is *C. coli* (10MF2039). The numbering indicates the nucleotide position within the *gyrA* gene of *C. jejuni*. Primer sites for GyrA1 and GyrA2 are shown by arrows indicating the direction of the oligonucleotide.

A comparison of the nucleotide sequences of the primers and their target sequences identified a number of mismatches, particularly between GyrA2 and its target (Fig 3.6)

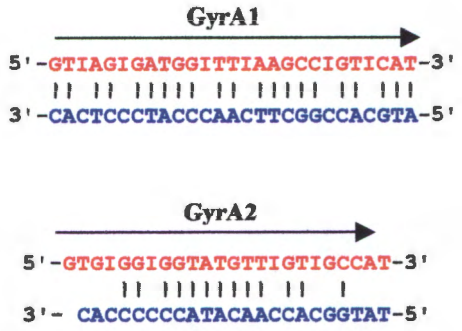


Figure 3.6 Alignment of primers, GyrA1 and GyrA2, to primer binding region of *C. coli* 10MF2039. Primer sequence is on the top line (red), bottom line (blue) is the corresponding binding region of 10MF2039.

These mismatches, particularly those in GyrA2, could account for the absence of amplicons. It has been suggested that 3-5 mismatches at 3'-ends will affect extension of the primer (Rolfs *et al.*, 1992).

Subsequent to obtaining this data, Zirnstein *et al.*, (2000) published sequences of primers that they had used to amplify QRDRs of fluoroquinolone resistant *C. coli*. Primers described by Zirnstein *et al.*, (2000) were used in further studies.

3.3.4 Amplification of *gyrA* Using Primers GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli4R

For this part of the study, samples were selected that represented a distribution of MICs. The samples used are shown in Table 3.4. Using the primers (GZ*gyrA*Ccoli3F and GZ*gyrA*Ccoli3F) described by Zirnstein *et al.*, (2000) a product of 505 bp was expected. No amplification product was obtained from *C. jejuni* DNA. However, products of the expected size were obtained with the DNA from the 7 isolates, including *C. coli* NCTC 11283 tested (Fig 3.7). The PCR products obtained from the pig isolates were purified [section 3.2.4] and sequenced directly [section 3.2.6].

Table 3.4 ***C. coli*** **isolates, and their corresponding ciprofloxacin MICs, used in the QRDR** ***gyrA*** **PCR assays.**

Sample	Ciprofloxacin Resistance	Ciprofloxacin MIC (μg/ml)
NCTC 11283	Sensitive	0.032
19S	Sensitive	0.032
53S	Sensitive	0.032
73R	Resistant (Intermediate)	2
9MF2038	Resistant	4
19R	Resistant	≥32
20R	Resistant	≥32

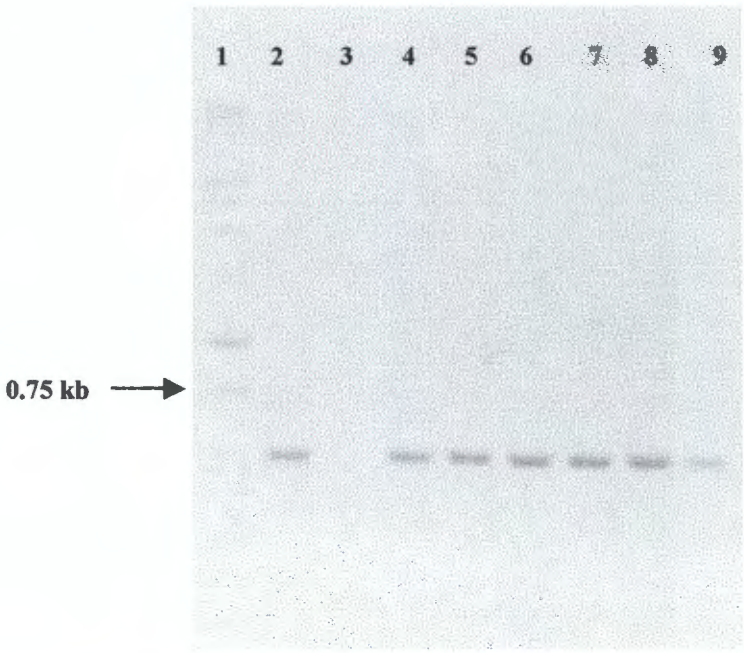


Figure 3.7 Amplification products obtained using ***GZgyrA*****Ccoli3F and** ***GZgyrA*****Ccoli4R.** Lane 1, Molecular weight marker 1 kb DNA ladder (Promega); lane 2, *C. coli* NCTC 11283; lane 3, *C. jejuni*; lane 4, 19R; lane 5, 19S; lane 6, 20R; lane 7, 53S; lane 8, 73R; lane 9, 9MF2038.

Nucleotide sequence data of the amplicons obtained from the isolates were aligned to each other, and to *C. coli* sequences in Gen Bank (Fig 3.8). Analysis of this data identified cytosine and thymine residues at base 260 in the sensitive and resistant strains, respectively. The presence of thymine instead of cytosine results in the incorporation of isoleucine rather than threonine at codon 86. One other difference can be seen at position 300. The sequences *C. coli* NCTC 11283 (sensitive) and *C. coli* 20R contain a thymine at base 300, whereas cytosine is in the corresponding position in the remaining sequences. As this does not result in an amino acid change, the mutation is silent.

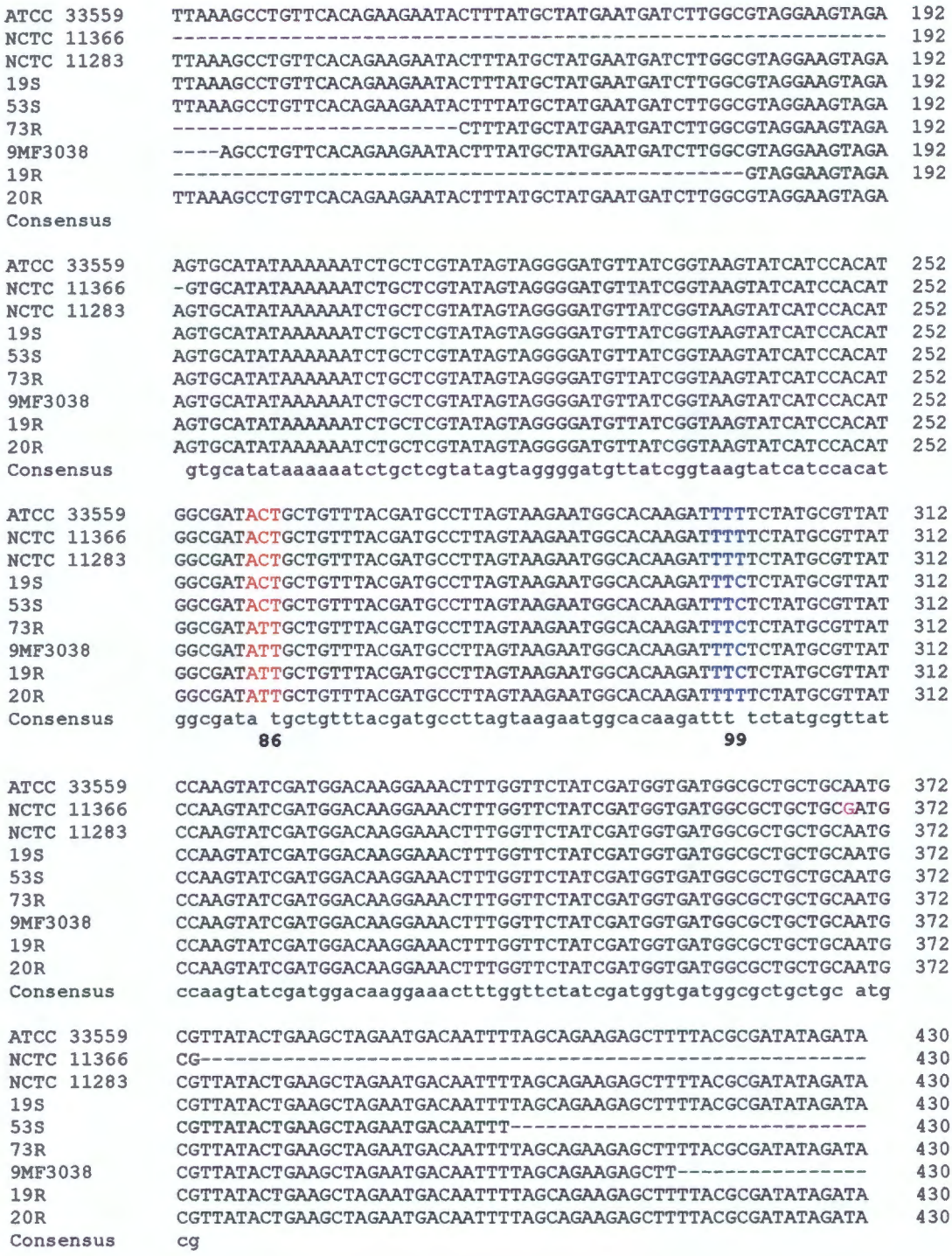


Figure 3.8 Nucleotide sequence alignment of partial *gyrA* gene sequences of *C. coli* isolates from this study and *C. coli* isolates in the GenBank database. The sequences of *C. coli* ATCC 33559 (accession number AF092101, Zirnstein *et al.*, (2000)), *C. coli* NCTC 11366 (accession number U63413) were compared to QRDR *gyrA* of *C. coli* NCTC 11283 (this study), and *C. coli* isolates 19S (MIC=0.032), 53S (MIC=0.032), 73R (MIC=2), 9MF2038 (MIC=4), 19R (MIC≥32), 20R (MIC≥32) this study. The consensus sequence is shown below. Red indicates codon 86 given in bold; blue indicates codon 99 given in bold.

3.3.5 Amplification of the QRDR *parC*

The single mutation of threonine to isoleucine at codon 86 in the GyrA protein may not be the whole explanation for the range of ciprofloxacin susceptibilities (Table 3.4). As high-level resistance (MIC of 125 μg/mL) has been shown to be associated with mutations in *gyrA* and *parC* in other organisms (Vila *et al.*, 1996; Pan & Fisher, 1999; Bush & Goldschmidt, 2000), the *parC* of resistant *C. coli* isolates was amplified.

Using the primers (P1 and P2) and assay conditions defined by Gibreel *et al.*, (1998) for the amplification of the *parC* QRDR of *C. jejuni*, no products were obtained with DNA from *C. jejuni* and *C. coli*.

In order to optimise the assay, numerous modifications were carried out:

3.3.5.1 Primers Titration

Using the primers at 0.1 μm, 0.5 μm, 1 μm and 1.5 μm, and the same conditions used earlier, no QRDR of *parC* was obtained from *C. jejuni* or *C. coli* DNA.

3.3.5.2 $MgCl_2$ Titration

Magnesium chloride was titrated across the following concentrations: 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 3.5 mM, 4 mM and 5 mM. No product was obtained with the DNA from *C. jejuni* and *C. coli*. At 5mM $MgCl_2$ and 1.5 μm primer, a product of the expected size was amplified, purified and sequenced. A comparison of the DNA sequence data with sequences in the data bank showed that it was similar to sequences in *Campylobacter* but not related to *parC*.

3.3.5.3 DNA Titration

DNA titration was carried out with the following concentrations: 100 ng, 200 ng, 300 ng, 400 ng, 800 ng and 1000 ng, using the conditions defined by Gibreel *et al.*, (1998). No product was obtained from *C. jejuni* and *C. coli* DNA.

3.3.5.4 Variation of Annealing Temperature

Annealing temperature was varied from 60°C-40°C using conditions specified by Gibreel *et al.*, (1998). No product was obtained from *C. jejuni* and *C. coli* DNA.

3.3.5.5 Design of New Primers

As a last resort, different primers (CjparCR2; Cj*parC*F2 & Cj*parC*R6) were designed based on the nucleotide sequence of the *parC* from *C. jejuni* (accession number Y18300) [Table 3.2; Fig 3.2].

Using primers, P1 and Cj*parC*R2, and the previous parameters described, no product was obtained with DNA from *C. jejuni* and *C. coli*.

Primer set, Cj*parC*F2 and Cj*parC*R6, were designed for the amplification of a 432 bp product. Using previously described conditions, no PCR product was obtained from *C. jejuni* DNA. A faint product of the expected size was observed from *C. coli* sample 19S: an agarose/DNA plug was taken and used as template in re-amplification. The product obtained was purified and sequenced. Once again, the DNA sequencing data showed that it was not related to *parC*.

3.4 DISCUSSION

There is cause for concern over the increasing number of *Campylobacter* isolates resistant to fluoroquinolones worldwide, following the introduction of fluoroquinolones into veterinary medicine (Endtz *et al.*, 1991). Fluoroquinolones are used for treatment of *Campylobacter* infections, thus increased resistance bears on the effectiveness of treatment for campylobacteriosis (Smith *et al.*, 1999). As pigs are a food source, they may be a source of resistant strains in humans. In this study, 57% (35/61) of the *C. coli* isolates were resistant to ciprofloxacin and nalidixic acid. In another study, 100% of *C. coli* isolates have been shown to be resistant to ciprofloxacin and nalidixic acid (Sáenz *et al.*, 2000).

A single Thr-86 to Ile change was identified in the GyrA proteins in the 4 resistant *C. coli* isolates studied. The GyrA protein for the sensitive strains did not contain this mutation, suggesting that the Thr-86 to Ile substitution is responsible for the increased resistance to fluoroquinolone antibiotics. This study supports recent work by Zirnstein *et al.* (2000) who also examined the *gyrA* QRDR of *C. coli* isolates and found that all ciprofloxacin resistant *C. coli* isolates had a Thr-86 to Ile mutation. Other studies on the GyrA protein of *C. jejuni* have shown that fluoroquinolone resistance is associated with a Thr-86 to Ile mutation in the GyrA protein (Wang *et al.*, 1993; Charvalos *et al.*, 1996; Gaunt & Piddock, 1996; Gibreel *et al.*, 1998; Ruiz *et al.*, 1998). Additional mutations in the *gyrA* of *C. jejuni* have been described by Wang *et al.* (1993). An Asp-90 to Asn and Ala-70 to Thr in two separate mutants resulted in moderate increases in the ciprofloxacin MIC: 4 μg/ml and 1 μg/ml, respectively.

In several other bacteria, mutations associated with quinolone resistance have been identified in both *gyrA* and *gyrB* genes. In *E. coli*, several mutations have been identified in the *gyrA* gene (Hallet & Maxwell, 1991; Vila *et al.*, 1994). However, it is the Thr-86 to Ile equivalent mutation in *E. coli* GyrA (Ser-83 to Ala) that is primarily responsible for fluoroquinolone resistance in *E. coli* (Hallet & Maxwell, 1991). Similar mutations have also been identified in quinolone resistant isolates of *Pseudomonas aeruginosa* (Kureishi *et al.*, 1994), *Staphylococcus aureus* (Sreedharan *et al.*, 1991; Goswitz *et al.*, 1992) and *Acinetobacter baumannii* (Vila *et al.*, 1995). However, in gram positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, topoisomerase IV is the primary target of fluoroquinolones (Pan & Fisher, 1996; Ferrero *et al.*, 1999).

A silent mutation was identified in the *gyrA* QRDR of fluoroquinolone resistant and susceptible *C. coli* isolates. *C. coli* NCTC 11283 and sample 20R possess a thymine at nucleotide 300, all other samples posses a cytosine at this position. Comparison with isolates from Zirnstein *et al* (2000), *C. coli* ATCC 33559 and *C. coli* NCTC 11366 show that these two isolates also possess a cytosine at this position. This difference at position 300 however does not result in a change at the amino acid level. Phenylalanine is the corresponding amino acid at codon 99.

Secondary mutations have been identified in the *parC* genes of organisms with high-level fluoroquinolone resistance (Vila *et al.*, 1996; Pan & Fisher, 1999; Bush & Goldschmidt, 2000). Whether secondary mutations in ParC are necessary for high-level resistance in *Campylobacter* spp. has not been extensively investigated. In one report an Arg-139 to Gln mutation in ParC of *C. jejuni* was identified but it could not account for variation in susceptibilities to ciprofloxacin (Gibreel *et al.*, 1998). In this study numerous attempts to amplify the *C. coli parC* gene were carried out, without success. Thus, whether mutations in *parC* explain the increased resistance (≥32 μg/ml) of some of the *C. coli* isolates is unknown. There is also the possibility that additional mutations in *gyrB*, or *parE* may influence the level of resistance (Gibreel *et al.*, 1998).

Other mechanisms of resistance in *C. coli* cannot be ruled out: mutations in GyrA combined with altered outer membrane proteins or efflux mechanisms may result in a modest increase in MIC (Cambau & Gutmann, 1993). Charvalos *et al.* (1995) have suggested that an energy-dependent efflux system with a broad specificity is associated with multidrug resistance in *C. jejuni*.

CHAPTER 4

SUMMARY & GENERAL CONCLUSION

Campylobacter coli and *C. jejuni* have been recognised as the leading causes of gastroenteritis worldwide (Tauxe, 1992). *C. coli* is the dominant species isolated from pigs while *C. jejuni* is commonly isolated from chickens. Fluoroquinolone resistance has been described in *Campylobacter* spp. (Piddock, 1995). In *C. jejuni* and *C. coli*, the incidence of fluoroquinolone resistance has increased steadily with reported rates as high as 88% in Spain, 84% in Thailand and 57% in Taiwan (Gallardo *et al.*, 1998; Hoge *et al.*, 1998; Li *et al.*, 1998; Saenz *et al.*, 2000). Contaminated food particularly poultry is the usual source of infection (Tauxe, 1992) and the presence of fluoroquinolone resistant strains in the food chain has raised concerns. Firstly, it has been shown that patients infected with resistant *C. jejuni* have a longer duration of diarrhoea compared to patients with fluoroquinolone sensitive isolates (Smith *et al.*, 1999). The second concern relates to the treatment of human infections. Although most cases of campylobacteriosis are self-limiting and do not require antibiotic treatment, severe and prolonged infections often require treatment. Fluoroquinolones are the treatment of choice for adults and are also prescribed for prophylactic treatment against traveller's diarrhoea (Taylor *et al.*, 1991; Nachamkin, 1993; Piddock, 1995). Treatment failure in immunocompromised patients with campylobacteriosis is cause for concern in that it may increase *Campylobacter*-associated deaths (Engberg *et al.*, 2001).

A total of 61 *C. coli* strains isolated from pigs were included in this study: 55 strains were obtained from pigs on farm A, while 6 strains were obtained from pigs on farm B, the source farm of pigs to farm A. Fifty-six percent (31/55) of strains from farm A were resistant to ciprofloxacin and nalidixic acid, while 67% (4/6) of strains from farm B were resistant to these antibiotics. Serotyping and *flaA* typing were carried out to study the epidemiology of the *C. coli* isolates. In addition, the molecular basis of the fluoroquinolone resistance was investigated.

The serotypes of 62% (38/61) of isolates were obtained, the remainder were non-typeable. Serotype O:24 (11/61) was the most frequent serotype identified, followed by O:5 (7/61). All O:24 and O:5 strains types were resistant to ciprofloxacin,

suggesting a relationship between these two serotypes and resistance, in the *C. coli* strains investigated. It is interesting to note that all O:5 strains had ciprofloxacin MICs of ≥32 μg/ml. Moreover, the *flaA* profile (profile 6) of the O:24 strains was unique to this serotype. Thus, in this instance there was perfect correlation between serotype and *flaA* profile. Serotypes O:48, O:54 and O:59 were identified in strains from both farms. RFLP analysis of the *flaA* gene revealed 13 distinct profiles in strains from farm A. Profile 1, including strains from farm A and farm B, was the commonest profile with 31% (17/55) of *flaA* typed strains in this profile. This profile was also observed in *C. coli* NCTC 11283. Two other profiles were common to both farms. These data suggest that some of the strains on the two farms are related. To be certain of the genetic identity of the isolates between farms it may be necessary to use an additional molecular typing scheme.

Resistant and sensitive isolates were isolated from 15 pigs on farm A. *flaA* profiles were obtained for 12 pairs, of which 4 pairs each had the same *flaA* profile, suggesting selection of resistant mutants of a previously sensitive population. In this context, selective pressure exerted by the use of fluoroquinolones may have played a role. This is supported by data showing that since the introduction of fluoroquinolones in to veterinary medicine, the incidence of antibiotic resistant strains has increased in Europe (Piddock, 1995).

No *flaA* product was amplified for 8% (5/61) of isolates. Investigation suggested a lack of complementarity between the primers and the target sequence at the 3' end. This data supports the findings of Harrington *et al.* (1997), who provided strong evidence of intragenomic recombination between the *flaA* and *flaB* genes of *C. jejuni*. It is possible that similar recombination events have occurred in the *flaA* gene of the PCR refractory *C. coli* isolates, resulting in a lack of complementarity between primers and binding target.

In *C. jejuni* and *C. coli*, it has been shown that a mutation of Thr-86 to Ile in the GyrA protein is associated with resistance to ciprofloxacin and nalidixic acid (Wang *et al.*, 1993; Zirnstein *et al.*, 2000). Although other mutations in GyrA have been described these do not play an influential role in resistance (Wang *et al.*, 1993). The mutation Thr-86 to Ile was identified in the GyrA protein of the resistant strains investigated in the study.

In addition to mutations in GyrA, Gibreel *et al.*, (1998) identified a mutation in ParC. Secondary mutations in *parC* have been associated with high-level fluoroquinolone resistance in other organisms such as *E. coli, S. pneumoniae* and *Enterobacter cloacae* (Vila *et al.*, 1996; Deguchi *et al.*, 1997; Pan & Fisher, 1999), although there was no convincing evidence that the mutation identified by Gibreel *et al.*, (1998) was associated with fluoroquinolone resistance. Many attempts were made to PCR amplify the QRDR of *parC* without success. Whether additional mutations in *parC* could account for the varying ciprofloxacin MICs for *C. coli* is unknown.

In conclusion, *C. coli* sensitive and resistant strains were isolated from pigs on two farms. RFLP *flaA* profiles suggested that although some of the isolates could be related, the majority of isolates were non-related. Serotype 24 and 5 strains were all resistant to ciprofloxacin and nalidixic acid, and profile 6 was unique to serotype 24. The profiles of paired resistant and sensitive isolates suggested that selection of resistant mutants from a previously sensitive population occurred, possibly by pressure exerted through use of fluoroquinolones. A mutation within the GyrA was associated with fluoroquinolone resistance.

APPENDIX A

Buffers, Media and Solutions

Ammonium Acetate (7.5M)

Ammomnium acetate	289g
Distilled water	200 ml

Store at 4°C.

Ampicillin (100 mg/ml)

Ampicillin	2 g
Distilled water	20 ml

The solution is filter sterilized and stored in aliquots at -20 °C. For selective plates ampicillin is added to the media at a final concentration of 100 µg/ml.

Chloroform-isoamylalcohol

The chloroform is mixed at a ratio of 24:1 with isoamylalcohol

EDTA (0.5 M)

EDTA	93.05 g
NaOH	10 g

The ingredients are dissolved in 400 ml of water and autoclaved.

Ethidium Bromide (EtBr)

Ethidium Bromide	0.1 g
Distilled Water	10 ml

Gel Tracking Dye (6×)

Bromophenol blue	25 mg
Sucrose	4 g
EDTA (0.5 M, pH 8.0)	0.4 ml
Distilled water	to 10 ml

Guanidium Thiocyanate Solution

Guanidium Thiocyanate	60 g
EDTA (0.5 M, pH 8.0)	20 ml
Distilled water	20 ml

The solution is heated to 65°C with mixing until dissolving. Solution is cooled and 5 ml of 10% (w/v) N-Lauryl-Sarcosine Sodium salt is added and made upto 100 ml with distilled water. Solution is filtered through a 0.45 μm pore.

IPTG (Isopropyl-β-D-thio-galactopyranoside)

IPTG	2 g
Distilled Water	8 ml

The solution is mixed and made up to 10 ml and frozen in 1 ml aliquots at -20°C. For selective plates, 50 μl IPTG is added to 100 ml agar.

Phenol

Commercial crystallized phenol	500 g
8-hydroxyquinoline	0.6 g
NaOH (2 M)	7.5 ml
Distilled water	130 ml
Tris-HCl (1 M, pH 7.6)	6 ml

The solution is liquefied at 40°C. The result is a solution of phenol in 10 mM Tris, pH 7.6.

Phosphate-Buffered Saline (PBS)

NaCl	6.8 g
Na_2HPO_4	3.5 g

Made upto 1000 ml, pH adjusted to 7.0.

Sodium Acetate (3 M)

Sodium acetate	204.05 g
Distilled water	400 ml

The pH is adjusted to 5.2 with glacial acetic acid and the volume made up to 500 ml. Stored at room temperature.

Sodium Hydroxide (10 N)

Sodium hydroxide	40 g
Distilled water	100 ml

Solution I

Tris-Cl (pH 8)	2.5 ml
EDTA (0.5 M, pH 8)	10 ml
Glucose (20% w/v)	5 ml
Distilled water	to 100 ml

Solution II

NaOH (10 N)	2 ml
SDS (20% w/V)	5 ml
Distilled water	to 100 ml

Solution III

Potassium acetate (5M)	60 ml
Glacial acetic acid	11.5 ml
Distilled water	to 100 ml

Store at 4°C.

SSC (10X)

$C_6H_5Na_3O_7.2H_2O$	0.15 M
NaCl	1.5 M

Tris-Acetate (TAE) Buffer (50×)

Tris	242 g
Glacial acetic acid	57.1 g
EDTA (0.5m, pH 8.0)	100 ml

The ingredients are mixed in distilled water to a final volume of 1000 ml, autoclaved and stored at room temperature.

To make 1X solution, the buffer is diluted 50 fold.

Tris-EDTA (TE) Buffer

Tris	10 mM
EDTA	1 mM

The pH is adjusted to 7.6.

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside)

X-gal is dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mg/ml. After the X-gal is dissolved, 1 volume of distilled water is added and the solution is stored at -20°C.

For selective plates, 0.5 ml X-gal solution is added to 100 ml agar.

2× YT Broth/Agar

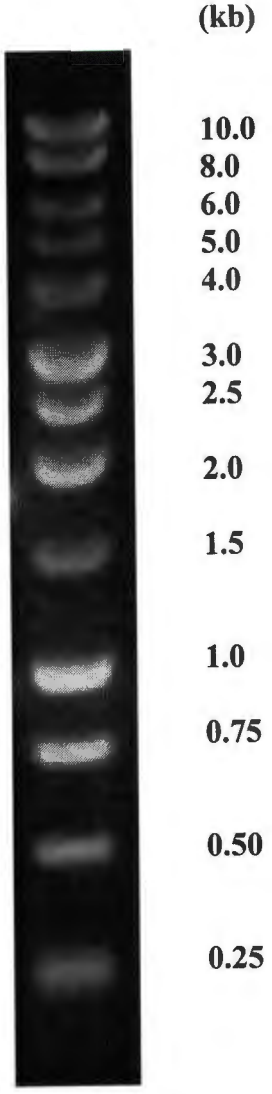
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Made up to 1000 ml with distilled water and autoclaved

For agar, 15 g is added.

APPENDIX B

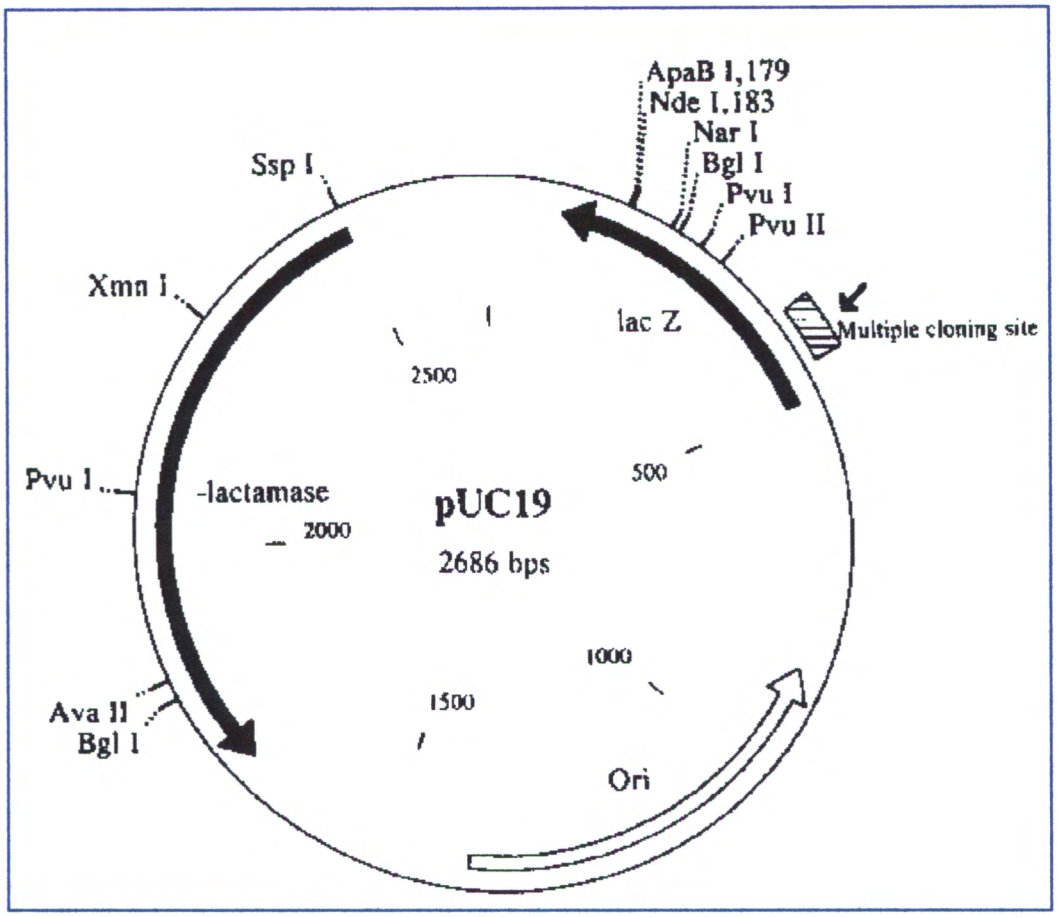
Molecular Weight Marker



Appendix B1: 1kb DNA Ladder (Promega).

APPENDIX C

Maps of Plasmids



Appendix C1: pUC19

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